HOST PROTEIN INCORPORATION IN HUMAN IMMUNODEFICIENCY VIRUS-1

By

Michael E. Linde, MS

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

September 2013

Abstract

Human Immunodeficiency Virus (HIV) incorporates a number of host proteins. These proteins can provide information on the function of viral proteins, as well as on the general process of HIV biogenesis. Determining the methods of incorporation and potential functional importance will help advance our knowledge of the HIV lifecycle and holds the potential to produce additional targets for antiretroviral therapy. Here, we used a variety of complementary techniques to determine which host proteins are incorporated into HIV particles. We found that most of the CD28 and B7 family costimulatory molecules are excluded from viral particles. Using a novel purification technique and mass spectrometry analyses, we were able to characterize host protein incorporation in HIV particles derived from CD4+ T-cell lines; we compared this data set to a reprocessed data set of monocyte-derived macrophages derived HIV-1 using the same bioinformatics pipeline. Seventy-nine clustered proteins were shared between the data sets. These clusters included an extensive collection of actin isoforms, HLA proteins, chaperones, ERM proteins, EH4, a phosphodiesterase, cyclophilin A, and others. As these proteins are incorporated in virions produced in both cell types, we hypothesize that these proteins may have direct interactions with viral proteins or may be important in the viral lifecycle. Additionally, this common protein set protein is predicted to interact with >1000 related proteins. Many of these secondary interacting proteins are reported to be incorporated into virions. Thus, only a few direct interactions between host and viral proteins may drive host protein composition in virions. We hypothesized that these may be driven by the tetraspanin family of proteins, putative membrane organizers determining the lipid and protein composition of tetraspanin enriched membranes. We

found that knockdown of various tetraspanins in T cell lines did not significantly alter viral release or phenotype. Ultimately, cell type-specific differences in host protein interaction and expression may drive virion phenotypic diversity, despite conserved primary viral protein-host protein interactions across cell types. Further, the primary interactions found between viral and host proteins are likely driven by selective pressures including response to host restriction factors and membrane structural requirements.

Advisor: James E.K. Hildreth

Readers: David R. Graham

ACKNOWLEDGEMENTS

While many people were vital during the course of my thesis work, this dissertation would not have come to pass without James E.K. Hildreth, MD, PhD and David R Graham, PhD. Their importance to me as mentors, colleagues, and friends cannot be overstated. It has been a true pleasure to have had the opportunity to work with both of them.

Many members of the Hildreth and Graham labs assisted, both physically and intellectually, on the work presented here. These include (in no particular order) Ihid Liao, PhD; Liao Zhang; Min Li; Edward Knapp, PhD; Craig Fletcher, DVM, PhD; Alvin George; Harry Taylor, PhD; Robin Broughton, PhD; Nkiruka Emiguiali, MD, PhD; Tiffany Walker, PhD; Ebony Coleman, Glenn Simmons, PhD; David Colqouhn, PhD and Ceereena Ubidiah Mohein. Other investigators, including Bindong Liu, PhD; Xinhong Dong, PhD; Waldemar Popik, PhD; and Donald Alecndor, PhD; as well as their lab members, were also helpful in discussions on this work. Mark Soloski, PhD and Chris Zink, DVM, PhD served on my thesis committee and were instrumental in this work. I would like to deeply thank everyone involved. I would also like to thank (and apologize to) anyone I may have omitted in this acknowledgment.

I would also like to thanks my partner, Susan, and my daughters, Miriam and Margot, for their patience and support.

TABLE OF CONTENTS

Abstract ii

Acknowledgements iv

Table of Contents v

List of Tables vi

List of Figures viii

Chapter 1: General Introduction 1

Chapter II: Human Immunodeficiency Virus-Type 1 Does Not Incorporate CD28 Family Member or Most B7 Family Members in the Viral Envelope 8

Introduction 9

Materials and Methods 11

Results 15

Discussion 28

Chapter III: The conserved set of host proteins incorporated into HIV-1 virions suggests a common egress pathway in multiple cell types 33

Introduction 34

Materials and Methods 37

Results 41

Discussion 56

Chapter IV: The role of tetraspanin proteins in host protein incorporation into the HIV-1 envelope 65

Introduction 66

Materials and Methods 71

Results.....75

Discussion.....102

Chapter V: Hypothetical Determinants of the HIV Envelop Composition 107

References.....115

Curriculum Vitae 132

LIST OF TABLES

Table 2-1. Flow cytometry-based phenotyping of HIV produces from H9 cells 27

Table 3-1. Reduced proteins in HIV-1 derived from CEMX174 cells following virion purification using DM or Optiprep 46

Table 3-2. Proteins able to differentiate between HIV-1 derived from CEMX174 cells or H9 cells 48

Table 3-3. Identified proteins common to MDM- and T-cell derived HIV-1 virions 52

Table 3-4. HIV-1-human protein interactions reported in the literature.....54

Table 5-1. Receptor Characteristics for Selected Enveloped Viruses 113

LIST OF FIGURES

Figure 2-1. Expression of select costimulatory markers in uninfected controls or chronically infected cell lines 16

Figure 2-2. Expression of costimulatory markers in chronically infected cell lines 18

Figure 2-3. Incorporation of costimulatory molecules into HIV-1 virions produced in chronically infected T cells or MDMs 20

Figure 2-4. Costimulatory molecule incorporation compared to cellular expression 22

Figure 2-5. Schematic of a novel flow-based phenotyping method to distinguish microvesicles from virions in mixed samples 25

Figure 3-1. Purification of HIV-1 virions using density modification 42

Figure 3-2. Comparison between the published MDM derived HIV-1 and other datasets 50

Figure 3-3. Hypothetical schematic of the impact of primary HIV-host protein interactions on virion phenotype 62

Figure 4-1. A dynamic view of the tetraspanin web 67

Figure 4-2. Phenotype of Jurkat/HIV_{JRCSF} cells following tetraspanins iRNA knockdown 76

Figure 4-3. Tetraspanin iRNA effect on p24 release in Jurkat/HIV_{JRCS}F cells 78

Figure 4-4. Tetraspanin iRNA effect on p24 release in PM1/HIV_{IIIB} cells 80

Figure 4-5. Effect of tetraspanin iRNA knockdown in Jurkat/HIV_{JRCSF} on HIV phenotype 82

Figure 4-6. Effect of tetraspanin iRNA knockdown in PM1/HIV_{IIIB} on HIV phenotype 84

Figure 4-7. Infectivity of HIV_{JRCSF} produced in iRNA treated cells 87

Figure 4-8. Selection against CD63 expression in mutagenized Jurkat cells 89

Figure 4-9. Subcloning of mutagenized Jurkat cells enriched for low cell surface CD63 92

Figure 4-10. Subclone analysis of surface and intracellular CD63 expression levels 94

Figure 4-11. Fluorescent microscopy of DR63 cells 96

Figure 4-12. Light microscopy of DR63 cells 98

Figure 4-13. HIV_{JRCSF} release from HIV-infected Jurkat and DR63 cells 100

Figure 4-14. A hypothetical mechanism for the incorporation of TEM-associated proteins into HIV-1 particles 103

CHAPTER I

GENERAL INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is one of the most devastating infectious disease epidemics in recorded human history, with an almost 35 million people estimated to have died from the disease. Further, an estimated 70 million people are believed to have been infected with Human Immunodeficiency Virus (HIV)-1 and -2, the virus that causes AIDS. Worldwide, there are approximately 34 million people living with HIV.[1] This epidemic, now 30 years old, continues to progress around the world, despite the fact that HIV infection is entirely avoidable and prevention methods have been known for almost the entire duration of the epidemic.

The HIV epidemic has also fostered one of the most significant scientific efforts in modern history, with the last 30 years of research on the scale equal to the development of atomic energy, manned space flight or the polio vaccination initiative. HIV research has produced an equally impressive result: the development of life-saving antiretroviral therapy. Antiretrovirals are now commercially available in several classes; nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, chemokine antagonists, and integrase strand transfer inhibitors (INSTIs). Notably, even though HIV requires a large number of cellular proteins to efficiently replicate, almost all of these options target viral proteins.

The 10kb HIV-1 genome encodes for 16 proteins, which are derived from the primary retroviral genes *gag*, *pol*, and *env*, and the accessory protein genes *tat*, *rev*, *nef*, *vpr*, *vpu*, and *vif*. In general, the accessory proteins serve to enhance viral replication, either

completing a vital step in viral lifecycle or by modulating the cellular environment to allow for greater replication. The rev protein is responsible for the transport of singly spliced or unspliced viral RNA transport to the cytoplasm.[2] Tat and nef are important for high levels of viral replication.[3,4] Vif binds the cytadine deaminase APOBEC3G and vpu binds tetherin, both interactions allowing for viral replication.[5,6] Vpr is involved in nuclear integration of the HIV pre-integration complex, cell cycle arrest, host-cell apoptosis, and immune suppression induction.[7-12] Notably, many of these proteins have been reported to have multiple functions, underscoring the complexity of HIV biology.

Antiretroviral therapy targets the classical retroviral proteins. NRTIs and NNRTIs inhibit reverse transcriptase, either through chain termination or active site inhibition. PIs inhibit protease function and, thus, proper formation of the viral core following viral budding. INSTIs prevent integration of the proviral DNA into the host genome. Fusion inhibitors bind the gp41 subunit of the viral envelope protein and prevent viral entry. CCR5 agonists prevent binding of the envelope protein to the CCR5 receptor and, therefore, block viral entry.[13]

While viral proteins are essential for proper viral replication, many host proteins are also involved in HIV replication. This has been demonstrated in viral budding, a complex process involving the co-ordination of many cellular and viral proteins.[14,15] Host proteins, such as the ESCRT family of proteins, proteins involved in ubiquitination, and proteins involved in proteasome function, have been shown to function in HIV budding.[16-18] Many of these proteins are also involved in the formation of exosomes, small vesicles

that bud into multivesicular bodies and are released from the cell.[19-24] Host proteins ensure that viral proteins reach their target membranes, allowing for formation of the nascent virion. HIV buds from lipid ordered domains (or detergent resistant membranes); targeting to this region of the cell membrane is dependent on the gag protein.[25-29] The HIV "lipidome" reflects budding from these regions. Brugger and colleagues quantitatively analyzed the lipid composition of producer cells and the progeny virus in the absence of detergent, finding that HIV-1 virions had similar composition to detergent resistant membranes and were enriched for lipids including cholesterol, sphingomyelin and dihydrosphingomyelin, plasmenylethanolamine, monohexosylceramide, saturated phosphatidyl choline species, and phosphotidyl serine.[30] The concentration of cholesterol and sphingolipids in these regions result in a tightly packed, ordered structure that is resistant to disruption by non-ionic detergents at low temperature.[31-33] Immunomicroscopy studies show that gag and viral envelope proteins colocalize with lipid raft markers on the surface of infected cells.[26,29] Proteins on the cytoplasmic side of the membrane, such as src family proteins and alpha subunits of G-proteins, are directed to these regions by the post translational addition of acyl groups; primarily myristylation or palmitylation.[34-36] Myristolation of the matrix domain of gag targets the protein to these regions.[28,37-40] In liposomes, gag strongly localizes to lipids with both acyl chains unsaturated over those with only one chain unsaturated. Cholesterol in the membrane region also leads to increased gag binding and closer phospholipid packing.[41] Interactions between gag and the cytoplasmic domain of env regulate the incorporation of the viral spike in the viral envelope.[42-48] It has also been reported that nef is involved in enriching

sphingomyelin and reducing polyunsaturated phosphatidylcholine species in the viral envelope.[49]

Budding from these domains appears to be crucial for the viral lifecycle. Several groups, including ours, have demonstrated that removal of cholesterol—a critical component of lipid ordered domains—from HIV particles results in inactivation. This appears to occur due to a loss of the ability to fuse to the target cell and the loss of virion integrity resulting in permeabilization of the virus.[50-56] While HIV has been shown to bud from detergent resistant membranes, the exact nature of the membranes is still unclear. For example, HIV has been shown to bud from "lipid rafts", but it has also been shown to bud from similar structures, tetraspanin enriched membranes (TEMs). While these two lipid ordered domains share many similarities, they are distinct membrane domains. However, Hogue and colleagues recently determined that the presence of gag induces the coalescence of lipid rafts and TEMs, and that this may be in a stepwise fashion.[57]

Further, the viral envelope presumably reflects the budding point, with the incorporation of many proteins found in detergent resistant membranes and the exclusion of proteins that do not localize to these domains.[26,58] These regions' rafts are highly enriched in GPI-anchored proteins, and generally exclude E cadherin and CD45.[59,60] A large number of proteins have been reported to be incorporated into the viral envelope, and these proteins belong to a number of different families or functional groups. For example, proteins involved in the immune response, including HLA molecules and some costimulatory molecules, are found in the viral envelope. Adhesion molecules, such as ICAM-1 and

LFA-1, are also reported to be in the viral envelope, as are cytoskeletal proteins, including actin. A short and incomplete list of proteins reported to be in the viral envelope includes annexin 2, Thy-1, CD44, CD46, CD55, CD59, CD63, and CD71. While many proteins have been reported to be incorporated into the viral envelope, it is important to consider that it is unlikely that all of these proteins are always incorporated into every viral particle. Thus, the host protein composition is likely to be heterogeneous, probably even between virions produced in the same cell. This is almost certainly the case for virions produced from different cell types. It is also important to note that these host proteins are not selected for in the viral envelope due to abundance in the plasma membrane—they are specifically enriched in lipid ordered domains.[58,61,62]

HIV-1 host protein incorporation is not just limited to membrane proteins. Cyclophilin A (CypA) has long been known to be incorporated into HIV-1 virions, facilitating HIV replication.[63-65] Notably, polymporphisms in CypA have been shown to affect disease progression.[66] Host proteins in the viral envelope may also affect HIV biology; virions incorporating the adhesion molecules LFA-1 and VLA-4 can have increased infectivity.[67] Thus, host protein incorporation can potentially affect disease course.

Here, we investigated host protein incorporation into the HIV virion, with an emphasis on membrane proteins that may have functional consequence, either on immune function or membrane fission/fusion. We also used proteomic analysis to identify common incorporated host proteins in HIV regardless of progenitor cell type. We believe the findings have implications on the method of host protein incorporation and provide a

model where a few HIV-host protein interactions serve as “hubs” and drive viral phenotype.

CHAPTER II

HUMAN IMMUNODEFICIENCY VIRUS-TYPE 1 DOES NOT INCORPORATE CD28 FAMILY MEMBER OR MOST B7 FAMILY MEMBERS IN THE VIRAL ENVELOPE

Introduction

HIV is known to incorporate many of the molecules found in the immunologic synapse, including adhesion and MHC class proteins. Lipid ordered domains have been shown to be important in the formation of the immunologic synapse;[68] HIV has also been shown to bud from lipid ordered domains.[26-28,54] Based on host molecule location, we hypothesized that HIV would incorporate costimulatory molecules, which are found in the immunologic synapse.

Esser and colleagues showed in 2001 that HIV derived from macrophages include CD80 (B71) and CD86 (B72), with CD86 incorporated in greater quantities.[69] However, a number of other B7 family members were discovered in macrophages around this time.[70] These include ICOS (Inducible T-cell Costimulator), PD-1 (Programed cell Death protein 1), PD-L1 (Programmed Death Ligand 1; B7-H1), PD-L2 (B7-H2), B7-H3 and B7-H4. Using an antibody against CD152 (CTLA-4), one group has been able to immunoprecipitate HIV-1 produced in Jurkat cells transfected with CD152. They were also able to immunoprecipitate HIV-1 with anti-CD28 and could block infectivity with antibodies directed against either of the CD28 family members.[71]

Costimulatory molecules of the B7 and CD28 families have important roles in regulating T cell activation, inhibition, and cytokine release profiles. HIV also infects CD4+ T cells and incorporates several of the host molecules found in the immunologic synapse in these cells. As prior studies had not investigated the potential for incorporation of then newly-discovered costimulatory proteins from CD4+ T cells, we endeavored to determine if

HIV indeed incorporates these molecules into the viral envelope. Further, we have also looked at the incorporation of host costimulatory molecules in the viral envelop for HIV-1 produced in both T cell lines and monocyte derived macrophages (MDM). As interaction of costimulatory molecules on with their cognate receptors on T cells results in signaling events that regulate immune responses, including the balance between Th1 and Th2 responses, it would be of interest if HIV incorporated costimulatory molecules in the viral envelope. This could have functional impacts and potentially impact disease progression.[72,73] Therefore, we investigated the incorporation of CD28 family members (CD28, ICOS, CD152 and PD-1) and well as B7 family members (CD80, CD86, B7-H1, B7-H2, B7-H3, and B7-H4). We have confirmed the incorporation of CD86 in virions produced in MDM, but were unable to confirm the incorporation of any other CD28 or B7 family member molecule in the HIV-1 envelope.

Materials and Methods

Viral stocks. Viral stocks were produced in chronically infected CD4+ T cell lines or primary MDMs. HIV_{MN} was produced in H9 cells; HIV_{RF} was produced in Jurkat cells and PM1 cells. Supernatant was depleted of microvesicles using CD45-coated paramagnetic beads (Dynal). Viral stocks were then pelleted through a 20% sucrose gradient at 100,000 x g for 1 hr and 15 minutes. Direct-pelleted BaL produced from MDM was purchased from ABI. Viral p24 content was assayed by ELISA, as previously described.[55]

Cell culture. All cells were propagated in RPMI supplemented with 10% FBS (HyClone), 100 μg/ml streptomycin, and 100 U/ml penicillin.

Immunoprecipitation. Viral particles were precipitated using *Staphylococcus aureus* Cowan strain (SaC; zysorbin). Primary antibodies were diluted in 3% BSA/PBS to 10 ug/mL. Viral stocks were diluted to 20-40 ng p24/mL. 100 μL diluted Ab and 100 μL diluted virus were incubated on ice for 60 minutes. Rabbit anti-mouse (RaM) IgG, Fc gamma fragment specific antibody was diluted to 1 mg/mL in 3% BSA/PBS. For each sample, 50uL SaC and 10 μL RaM were incubated on ice for 30 minutes. 60 uL SaC/RaM was added to each sample of virus/primary antibody and mixed on ice for 60 minutes. Samples were spun at 2700 RPM at 4ºC for 7 minutes. Supernatant was saved for analysis. The SaC/sample mixture was washed 2-times with 3 mL 10X PBS and one time with 1X-PBS. Each sample was lysed with 700 μL lysis buffer (TEN pH 8.0, 1% triton X-100) and incubated on ice for 45 minutes. SaC was pelleted at 32,000 RPM for 2

minutes and 650 µL of supernatant was saved. Remaining SaC from the supernatant was pelleted and 600 µL of supernatant was saved for analysis. Supernatant was serial diluted (1:2) and plated for analysis by HIV p24 ELISA.

HIV p24 ELISA. GagM1 (Leukocyte Immunochemistry Laboratory; LIL) antibody was diluted to 10 µL/mL n 50 mM TRIS pH 9.5. For each well of CoStar Fast binding ELISA plates, 100 µL of diluted GagM1 antibody was added and plates were incubated overnight at room temperature. Antibody was removed and 200 µL 3% BSA/PBS was added to each well. Plates were incubated 2 hours at 37ºC. Plates were washed 8-times with PBS/0.05% Tween 20. p24 standards were diluted to 1000, 250, 125, 62.5 and 31.2 pg/mL in standard antigen dilution buffer (RPMI-1640, 10% FCS, 1.0% Triton X-100). 100 uL of sample per well was added to the plates in triplicate, along with p24 standards. Plates were incubated overnight at room temperature and then washed 8-times with PBS/0.05% Tween 20. Biotinylated anti-p24 polyclonal IgG was added to each well (100 µL at 0.1 mg/mL) and incubated for 2 hours at room temperature. Plates were washed 8-times with PBS/0.05% Tween 20. 100 uL of streptavidin-peroxidase (1:10,000 in 5% NGS, 1% BSA, PBS, 0.05% Tween 20) was added to each well and incubated for 30 minutes at room temperature. Plates were washed 8-times with PBS/0.05% Tween 20. 100 µL of substrate (10 ml Na Acetate/Citrate, 100 µL TMB, 5 µL H_2O_2) was added to each well and incubated for 20 minutes at room temperature. The reaction was stopped with 50 µL/well of 0.5 M H_2SO_4 and plates were read at 450 nm on a 750 Microplate Reader.

Flow Cytometry. Flow cytometry was performed by first fixing cells with phosphate-buffered saline (PBS) containing 2% paraformaldehyde and then permeabilizing cells with PBS buffer containing 1% BSA, 5% normal goat serum (NGS), and 0.2% saponin, followed by staining with appropriate antibodies (Abs). Infection of cells was determined by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated anti-p24 (Coulter). The primary MAbs were detected with Alexa Fluor 647-conjugated goat anti-mouse polyclonal antibody. Virus production was measured by anti-p24 gag enzyme-linked immunosorbent assay (ELISA). Compensation was performed with single-stained cells. Data were collected on FACSCalibur (BD Biosciences) instruments and analyzed with FACSDiva (BD Biosciences) or Flowjo (Tree Star) software. For immunofluorescence staining, cells were fixed with 2% paraformaldehyde in PBS and permeabilized with PBS containing 5% NGS, 1% BSA, and 0.25% Triton X-100. Cells were than stained with indicated primary and secondary Abs. At the time of viral production, flow cytometric analyses were conducted to determine the phenotype of cells used to produce virus. A minimum of 10,000 cells were run on a Beckton Dickenson FACScaliber.

Flow cytometric based analysis of HIV phenotype. Viral and microvesicle membranes were labeled with the fluorescent dye PKH67 as follow. Virus/microvesicles (10 μg total protein) was resuspended in 1 ml of diluent C in an ultracentrifuge tube and mixed with PKH67 in diluent C (final concentration $5x10^6$ M) and incubated for 3 min. The ultracentrifuge tube was filled with 0.1% BSA/PBS and spun for 65 minutes at 100,000 ×

g. Samples were washed twice in 0.1% BSA/PBS and resuspended in 250 ul 0.1% BSA/PBS. Total protein was quantified by BCA assay.

Beads were prepared as follows: 25 μL anti-mouse IgG beads were incubated with 5 μL primary Ab in 0.1% BSA/PBS in total volume of 50 μL and rotated overnight at 4º C. Beads were washed twice with 0.1% BSA/PBS (2-3mL). Beads and antibodies were cross-linked with 2% PFA for 15 minutes at room temperature and then washed with 0.1% BSA/PBS. Beads were blocked in 5% NMS/PBS (15 minutes at room temperature) and then washed with 0.1% BSA/PBS. Beads were resuspended in 100 μL 0.1% BSA/PBS.

Conjugated beads (2 μL) were incubated with virus/microvesicles (10 μg total protein) in total volume of 20 μL and rotated for 2 hours at room temperature. Beads were washed 2-times with 0.1% BSA/PBS. Beads were incubated with 50 μL PFA at room temperature for 15 minutes and washed twice with 0.1% BSA/PBS. Virus/microvesicles were incubated with 1 μg anti-gp41-biotin in a total volume of 50 μL (5% NMS), rotated in the dark for 1 hour at room temperature. Samples were then washed with 0.1% BSA/PBS and incubated with 10 μL SA-PE for 45 minutes in dark at room temperature. Samples were washed twice with 0.1% BSA/PBS and resuspended in 250 μL 2% PFA for 15 minutes, washed, and then 250 μL 0.1% BSA/PBS. Samples were then analyzed on a FACScalibur for FITC and PE levels.

Results

Producer cell lines contain costimulatory molecules

Phenotyping cell lines by flow cytometry, we observed high levels of CD45, CD152, and B7-H2 in multiple different T cell lines, both uninfected and chronically HIV-1 infected (Figure 2-1). Lower levels were seen for CD80, ICOS, B7H1, and PD1. We also saw significant shifts in mean fluorescent intensity (MFI) in chronically infected T cell clones (Figure 2-2). MFI shifts were observed in at least one cell type studied for all costimulatory molecules assessed. Thus, there was sufficient evidence to support phenotyping of viral particles for these costimulatory molecules.

HIV-1 virions do not incorporate most costimulatory molecules

We then assessed incorporation of costimulatory molecules and control molecules in HIV-1 virions. As shown in Figure 2-3, we immunoprecipitated virions with primary antibodies against B7-H1, B7-H2, B7-H3, B7-H4, ICOS, B7RP1 (ICOS ligand), CD80, CD86, CD152, and PD-1. We also used the positive controls gp41 and HLA A/B/C and the negative controls IgG1 (isotype control) and CD45. We saw no incorporation above the negative controls for almost all molecules studies, except for CD86, which was present in virions produced from MDMs. We also compared molecule incorporation to host cell expression for each marker (Figure 2-4). While costimulatory molecules were expressed in many cases, we were not able to immunopreciptate viral particles using antibodies against these costimulatory molecules. Notably, antibodies against positive controls, HLA molecules and gp41, were able to immunopreciptate virions.

Figure 2-1. Expression of select costimulatory markers in uninfected controls or chronically infected cell lines.

Cell lines were assessed for costimulatory molecule expression by flow cytometry. The percent of positive cells are indicated. Isotype controls (IgG1) and phenotype controls (CD45) are also shown.

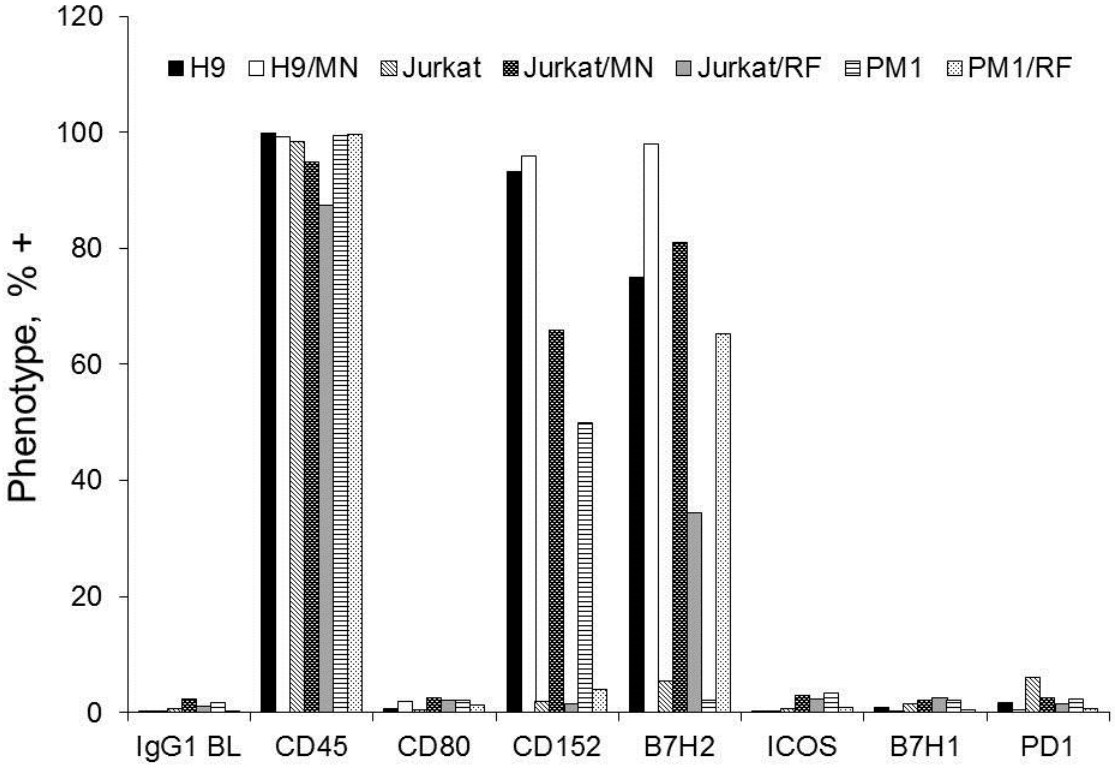

120
100
80
60
40
20
0
Phenotype, % +
H9
H9/MN
Jurkat
Jurkat/MN
Jurkat/RF
PM1
PM1/RF
IgG1 BL
CD45
CD80
CD152
B7H2
ICOS
B7H1
PD1

Figure 2-2. Expression of costimulatory markers in chronically infected cell lines.

Cell lines were assessed for costimulatory molecule expression by flow cytometry.

Representative histograms are shown.

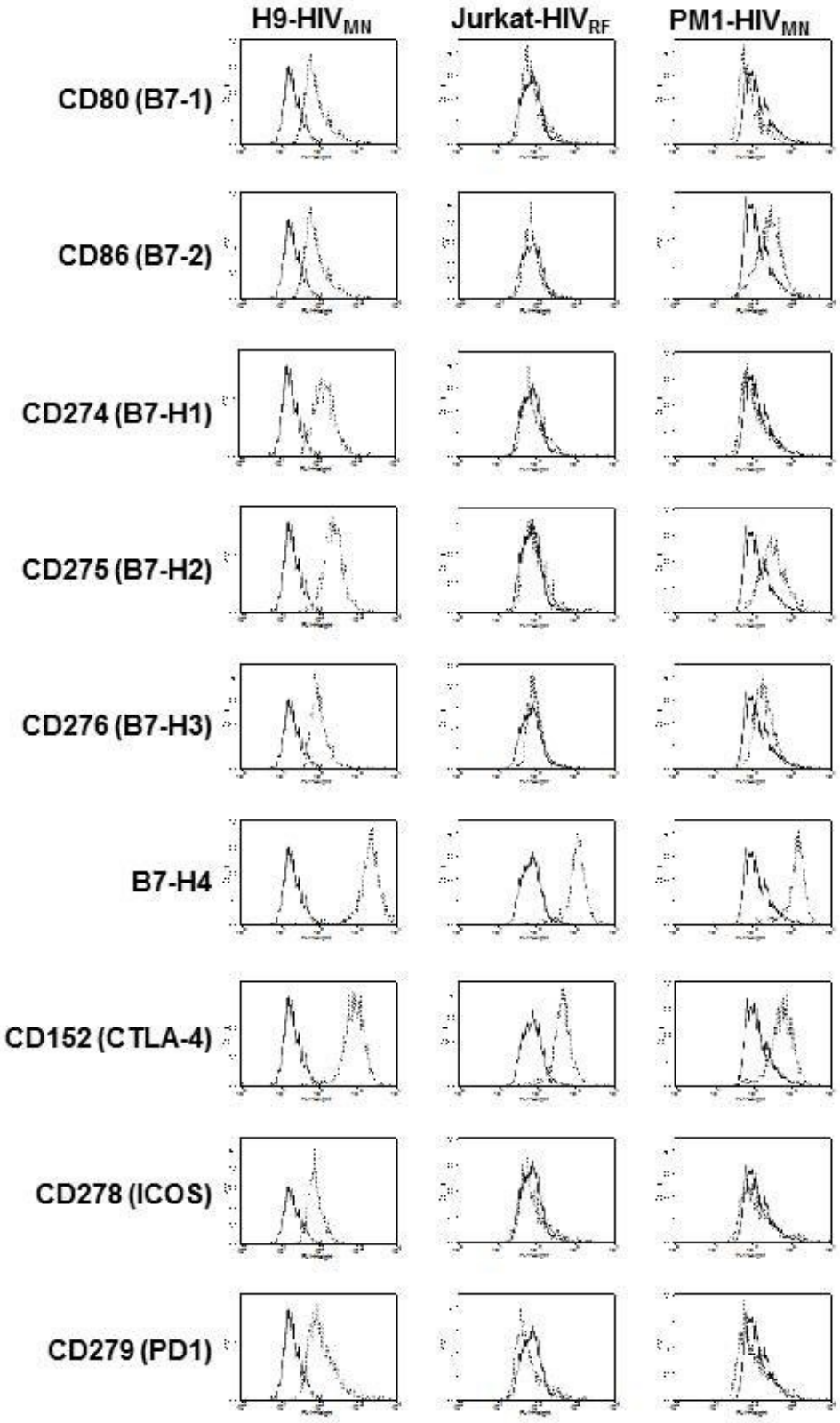

H9-HIV$_{MN}$
Jurkat-HIV$_{RF}$
PM1-HIV$_{MN}$
CD80 (B7-1)
CD86 (B7-2)
CD274 (B7-H1)
CD275 (B7-H2)
CD276 (B7-H3)
B7-H4
CD152 (CTLA-4)
CD278 (ICOS)
CD279 (PD1)

Figure 2-3. Incorporation of costimulatory molecules into HIV-1 virions produced in chronically infected T cells or MDMs.

Virions from H9/HIV$_{MN}$, Jurkat/HIV$_{RF}$, PM1/HIV$_{RF}$, and MDM/HIV$_{BaL}$ were immunoprecipitated with antibodies against multiple costimulatory molecules or control molecules. Results are from three independent experiments.

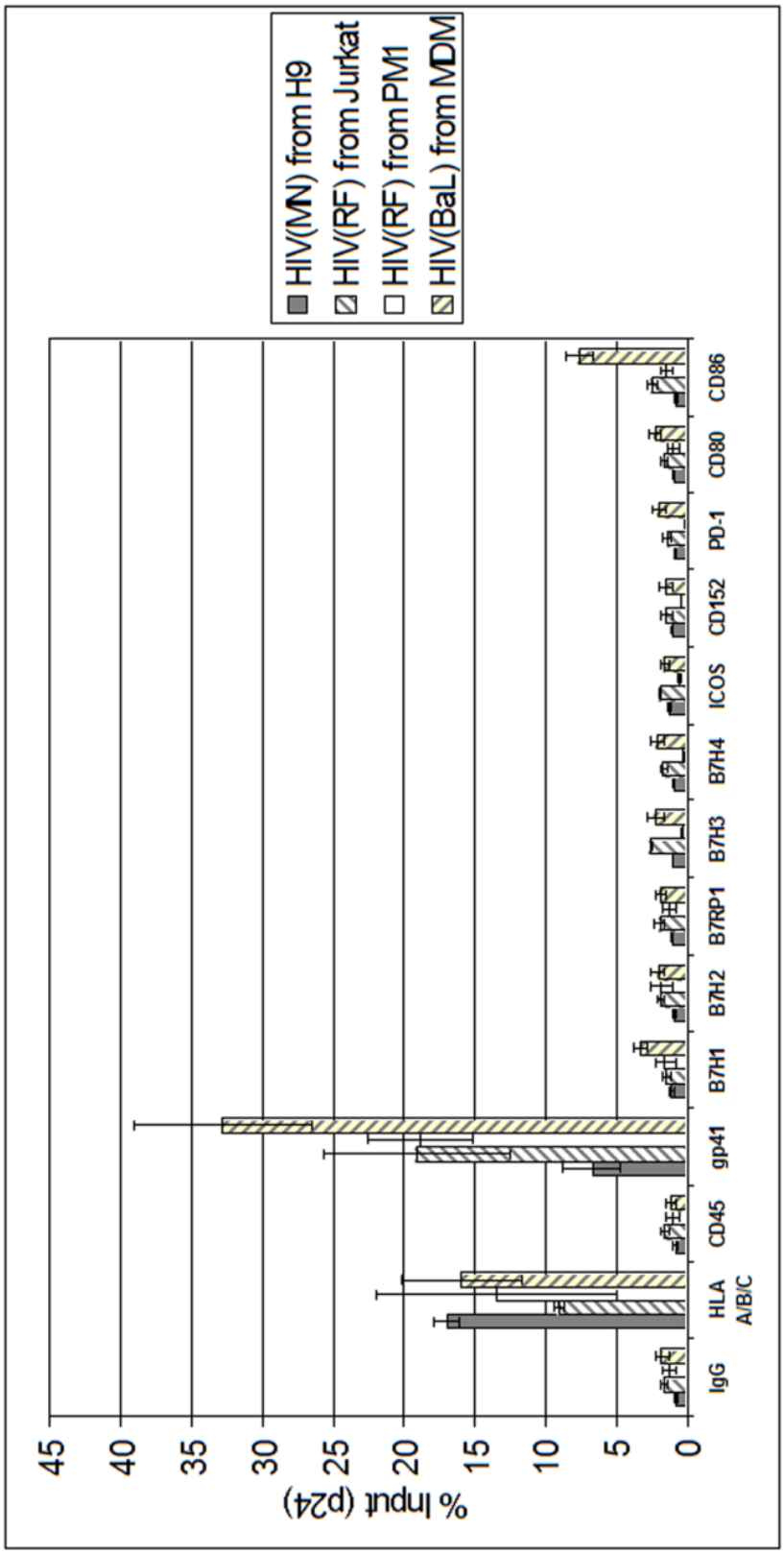
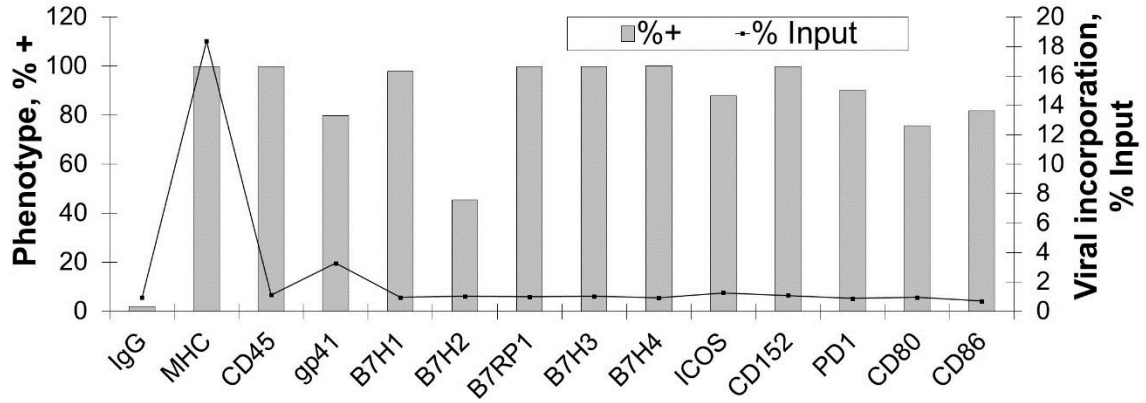
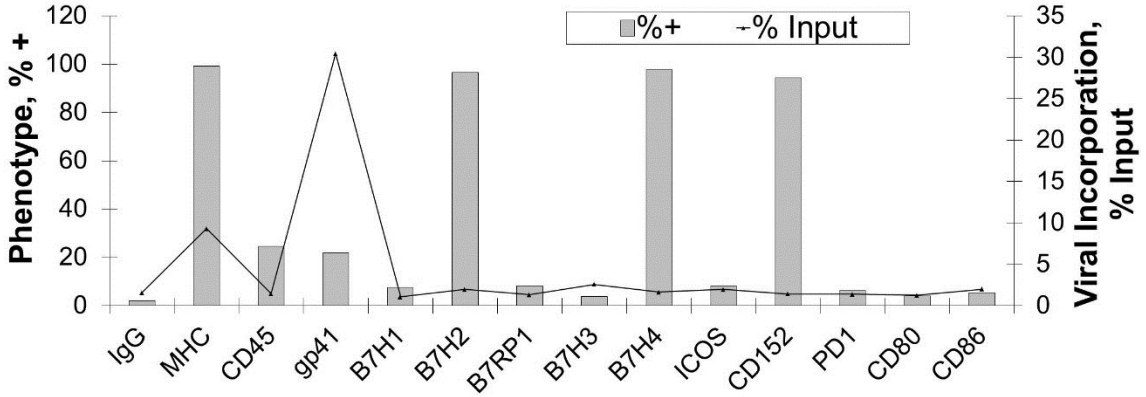

HIV(MN) from H9
HIV(RF) from Jurkat
HIV(RF) from PM1
HIV(BaL) from MDM
% Input (p24)
0
5
10
15
20
25
30
35
40
45
IgG
HLA A/B/C
CD45
gp41
B7H1
B7H2
B7RP1
B7H3
B7H4
ICOS
CD152
PD-1
CD80
CD86

Figure 2-4. Costimulatory molecule incorporation compared to cellular expression. Incorporation of costimulatory molecules was compared to the producer cell expression for each marker. (A) H9/MN; (B) Jurkat/RF; (C) PM1/RF. MHC = HLA A/B/C.

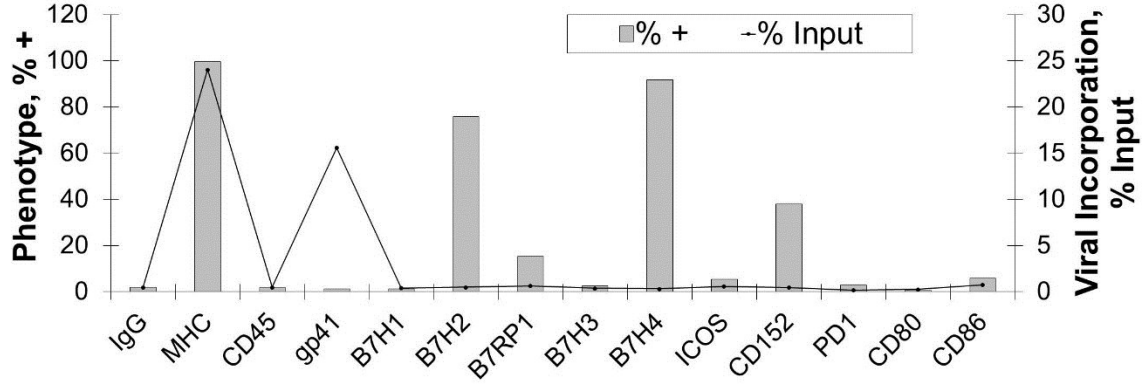
A


%+
% Input
Phenotype, % +
Viral incorporation, % Input
120
100
80
60
40
20
0
20
18
16
14
12
10
8
6
4
2
0
IgG
MHC
CD45
gp41
B7H1
B7H2
B7RP1
B7H3
B7H4
ICOS
CD152
PD1
CD80
CD86

B


%+
% Input
Phenotype, % +
Viral Incorporation, % Input
120
100
80
60
40
20
0
35
30
25
20
15
10
5
0
IgG
MHC
CD45
gp41
B7H1
B7H2
B7RP1
B7H3
B7H4
ICOS
CD152
PD1
CD80
CD86

C


% +
% Input
Phenotype, % +
Viral Incorporation, % Input
120
100
80
60
40
20
0
30
25
20
15
10
5
0
IgG
MHC
CD45
gp41
B7H1
B7H2
B7RP1
B7H3
B7H4
ICOS
CD152
PD1
CD80
CD86

In order to further confirm the presence or absence of host-derived surface markers on HIV virions, we developed a novel flow cytometry-based assay to phenotype viral particles. In this assay, virions were labeled with the fluorescent membrane dye PKH67 and immunoprecipitated with microbeads specific for membrane proteins. The beads were then run through a flow cytometer, with the FITC channel showing the amount of microvesicles attached to the microbeads. In order to rule out microvesicle contamination, the microvesicle coated microbeads were incubated with an anti-gp41-PE antibody. Thus, FITC- and PE-positive populations represented viral particles, while FITC-only populations represented microvesicles (Figure 2-5).

As shown in Table 2-1, we were unable to effectively discriminate between viral particles and microvesicles using this method. However, we were able to capture and assess the FITC+ population, representing both microvesicles and virions. We observed significantly higher FITC levels when capturing using HLA A/B/C, CD81, or CD45 compared to isotype controls. We were unable to see significant differences in capture using two separate CD152 antibodies or antibody against CD80.

Figure 2-5. Schematic of a novel flow-based phenotyping method to distinguish microvesicles from virions in mixed samples.

Microbeads were coated with a pan anti-mouse IgG, which was then conjugated to a mouse anti-human primary antibody. Microvesicles and HIV virions were labeled using the membrane dye PKH67. Microvesicles and virions were immunopreciptated and further mixed with anti-gp41-PE. Samples were analyzed by flow cytometry, gating on bead populations. FITC-positive, PE-negative populations are indicative of microvesicles, while FITC/PE-positive populations are indicative of virions.

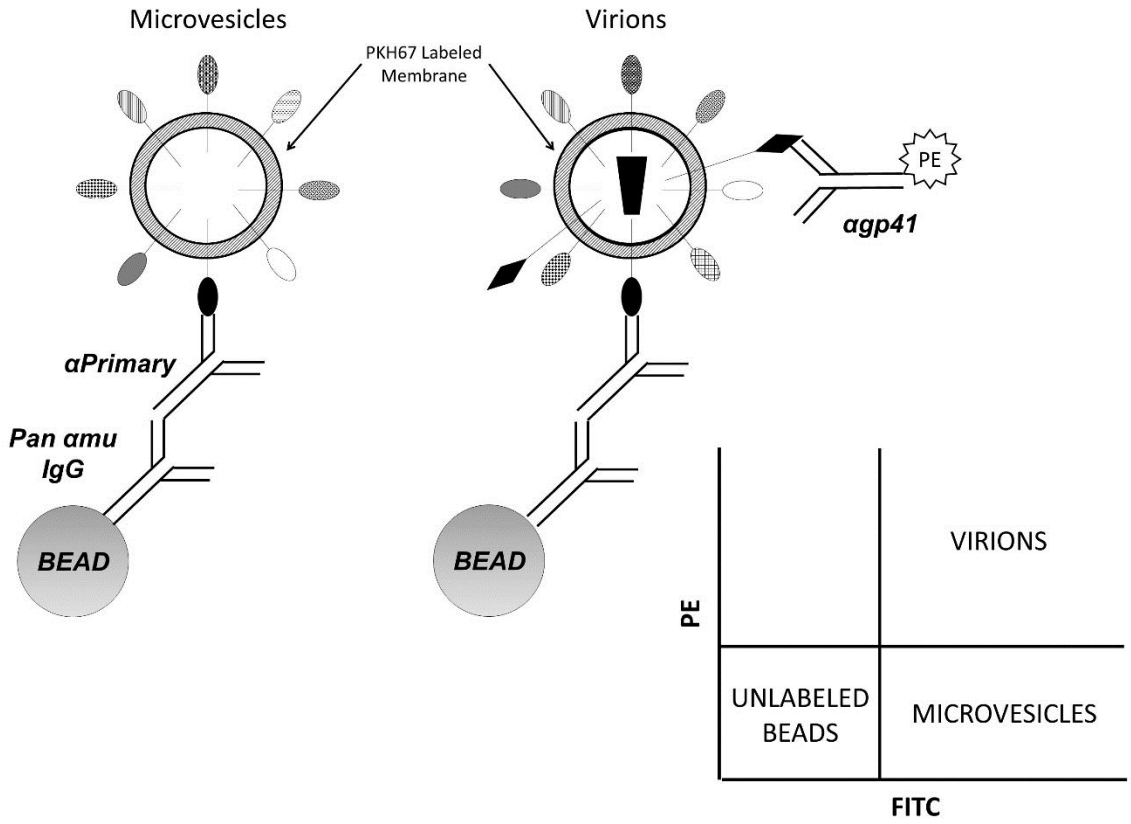

Microvesicles
Virions
PKH67 Labeled Membrane
PE
αgp41
αPrimary
Pan αmu IgG
BEAD
BEAD
VIRIONS
PE
UNLABELED BEADS
MICROVESICLES
FITC

Table 2-1. Flow cytometry-based phenotyping of HIV produces from H9 cells.

	FITC-/PE- (% +)	FITC-/PE+ (% +)	FITC+ /PE+ (% +)	FITC+/PE+ (% +)
IgG	99.63	0.03	0.29	0.06
HLA A/B/C	46.39	0.05	53.48	0.08
CD81	27.45	0.05	72.42	0.08
CD45	50.69	0.00	49.25	0.05
CD152 (BD)	98.51	0.05	1.43	0.00
CD152 (LIL)	99.11	0.02	0.85	0.02
CD80	97.05	0.05	2.21	0.69

BD = Becton Dickenson; LIL = Leukocyte immunochemistry laboratory (Hildreth laboratory)

Discussion

Here, we did not find evidence supporting the incorporation of most known costimulatory molecules in the viral envelope. It has previously been reported that CD80 and CD86 are incorporated into the viral envelope, with CD86 incorporated to a greater degree than CD80.[69] Our data supports this prior observation. However, other B7 family members, including B7-H1, B7-H2, B7-H3, and B7-H4, were not observed in the viral envelope.

We also did not find any evidence for incorporation of CTLA-4 in the viral envelope. This is notable considering the incorporation of CTLA-4 in the viral envelope has been published in the *Journal of Infectious Diseases*.[71] Given that CTLA-4 incorporation has been published in a peer reviewed journal, we tried to assess incorporation under different conditions and with several techniques. Using a standard immunoprecipitation technique with *Staphylococcus aureus* protein A as the precipitant, we were unable to pull down significant amounts of virus with three separate CTLA-4 antibodies (one commercial and two developed in our laboratory). This was true for many viral strains produced in many different cell lines. Further, we were unable to find any evidence of CTLA-4 incorporation in virus produced from activated PBMCs (data not shown) or in commercially-purified virus produced from MDMs. Western blots of concentrated lysed viral stocks also failed to show any evidence of CTLA-4 or other costimulatory markers (data not shown). We were unable to confirm CTLA-4 in the viral envelope in viral stocks produced from 293T cells cotransfected with proviral clones and CTLA-4 expression plasmids (data not shown). Finally, using a novel flow cytometry-based assay, we were also unable to detect the presence of CTLA-4 in the viral envelope or in

microvesicles. Additionally, proteomic analyses of host proteins in HIV virions have not detected the presence of CTLA-4 or any other costimulatory molecules (see Chapter III).[74] These data together very strongly indicate that, contrary to the published literature, CTLA-4 is not incorporated to any significant degree in the viral envelope.

The lack of CTLA-4 and other costimulatory markers in the viral envelope has some significant implications on hypotheses that HIV can function as an antigen presenting cell. HIV has been shown to incorporate a number of proteins that are found in the immunologic synapse, including MHC molecules, CD86, and ICAM.[69,75-77] The incorporation of adhesion molecules has been shown to have a functional significance for the virus, aiding in attachment to cell membranes.[67] The inclusion of MHC molecules has been suggested to potentially serve as one signal for the activation of CD4+ cells by HIV. As HIV infects actively replicating CD4 cells, activation of quiescent cells upon viral attachment would have the potential to be beneficial for the virus. Further, as MHC molecules in the viral envelope would likely have viral antigen in their clefts, HIV would be posited to preferentially infect HIV-specific T cells. This has been shown to be the case by Douek and others.[78-80] However, this line of reasoning requires that the HIV envelope contains molecules capable of delivering a costimulatory signal. For the activation of T cells, this would require CD80 or CD86, both of which are incorporated to varying degrees in the viral envelope.[69] In 2004, Giguere and colleagues reported that inclusion of CD80 or CD86 in the viral envelope results in NF-κB induction, supporting this hypothesis.[81] This same group has suggested that HIV acts as an antigen presenting cell, which may help explain preferential infection of HIV-specific CD4+ T cells.[82]

Activation induced by host proteins incorporated in the viral envelope could have a significant effect on HIV pathogenesis, as chronic activation is proposed to result in immune exhaustion and the development of AIDS in people with uncontrolled HIV infection.[83] However, activation or suppression effects that may be predicted from the incorporation of other B7 or CD28 family members in the viral envelope are unlikely, due to their lack of inclusion in virions produced in either lymphocytes or monocytic cells.

The lack of CTLA-4 in the viral envelope has some additional significance. One of the primary features of HIV infection is an increase in anergic T cells. One mechanism of T cell anergy is though the B7-CTLA-4 interaction. In this interaction, there is an inhibitory signal cascade sent through CTLA-4 in T cells, which prevents continued activation of cells. However, there is also a corresponding signal cascade in monocytic cell lines though B7 molecules, which induces tryptophan metabolism and results in localized micronutrient depletion. This induces an anergic response in T cells surrounding the monocytic cell.[72,73] While we originally hypothesized that HIV-1 virions might be capable of inducing anergy upon incorporation of CTLA-4 in the viral envelope, it appears that this is not the mechanism.

HIV has been shown to affect costimulatory expression within cells infected with HIV or chronic infection with other viruses. For example, LCMV-infected mice show upregulated PD-1 and CD152 in exhausted cells. Notably, blockade of PD-1 interaction with one cognate ligand, B7-H1 (PD-L1), restores CD8+ T cell function in these mice.[84]

Similarly, PD-1 is upregulated in chronic HIV infection, with similar impacts on CD4 and CD8 responses.[85,86] Accordingly, blockade of this interaction has been suggested a potential therapeutic target in chronic viral infections.[87-89] CD152 has also been shown to be upregulated in HIV and SIV infection, which has been suggested to lead to increased tryptophan catabolism and associated anergy.[90,91] Moreover, HIV-specific T cells show CD152 upregulation and this correlates with immune dysfunction in HIV-positive subjects.[92] Further, B7-H1 has been shown to be upregulated in HIV infection, while CD86 is downregulated, which could produce similar inhibitory effects.[93,94] While changes in costimulatory molecule expression can—and likely are—a mechanism of immune dysfunction in HIV and other chronic viral infections, it is unlikely that these changes are resultant from any inclusion of costimulatory molecules in the viral envelope.

We investigated a number of different viral strains and different cell type, which has importance as the host protein composition in the viral envelope can vary depending on the viral strain and cell type.[75,95,96] This intuitively makes sense, as changes in viral genetic sequences and corresponding protein changes have the potential to affect host-viral protein interactions. Incorporation may be impacted by producer cell differences in costimulatory molecule localization. Of particular note, while MHC molecules and costimulatory molecules are localized to lipid ordered domains in both CD4+ T cells and monocytic cells, MHC Class II and CD28 family member localization in CD4+ cells is found at sites of antigen presenting cell (APC)-T cell interaction, while MHC Class II and B7 family member localization is not polarized in monocytic cells.[97,98] This may

account for the observed lack of costimulatory molecule inclusion in virions purified from cell culture supernatant in CD4+ T cells. It is possible that HIV may be capable of incorporating CD28 family members *in vivo*, but these virions would bud directly into an engaged immunologic synapse and, therefore, may not be found to a significant degree in circulation.

Additionally, the host-viral interactions will likely depend on the protein composition of the producer cell; however, it appears that some host proteins are incorporated into HIV virions regardless of producer cell type.[96] This aspect of host-viral protein interactions and resultant host protein incorporation is further explored in the next chapter "The conserved set of host proteins incorporated into HIV-1 virions suggests a common egress pathway in multiple cell types."

CHAPTER III

THE CONSERVED SET OF HOST PROTEINS INCORPORATED INTO HIV-1 VIRIONS SUGGESTS A COMMON EGRESS PATHWAY IN MULTIPLE CELL TYPES

The work contained in this chapter has been published previously in:

Linde ME, Colquhoun DR, Mohien CU, Kole T, Aquino CV, Cotter R, Edwards N, Hildreth JE, Graham DR. The conserved set of host proteins incorporated into HIV-1 virions suggests a common egress pathway in multiple cell types. *J Proteome Res*. 2013;12(5):2045-54.

Introduction

During HIV replication and packaging, HIV relies on the coordinated interactions between viral and host proteins.[99] As HIV buds, it incorporates hundreds of cellular host proteins into the nascent virion, either into its lipid bilayer or inside the HIV virion.[74,76,100] Several studies have indicated that host protein incorporation affects both HIV attachment and infectivity.[67,101] Other proteins, such as cyclophilin A, have been implicated in the HIV lifecycle;[63-65,67,76] however, due to the large number of host proteins reported to be incorporated into HIV virions, it is difficult to determine the biological relevance, if any, of many of these proteins.

We hypothesized that host proteins that play a significant role in the HIV-1 virion lifecycle or those that significantly affect HIV spread through the host would be conserved in the virus regardless of the progenitor cell type. HIV-1 infects multiple cell types, most prominently macrophages and CD4+ T cells. As these cell types have different protein expression patterns and surface protein composition, it is expected that HIV-1 virions budding from these different cell types carry different sets of host proteins. Further, it is likely that many of the proteins incorporated by the virus are done so through secondary or higher interactions. Here, we define a minimal set of relevant host proteins that are incorporated into HIV-1 virions from multiple cell types.

Mass spectrometry (MS) analysis of purified viral particles is one tool for determining which host proteins are incorporated in HIV virions on a global scale. While there are biochemical and proteomic techniques that can be used to identify HIV-associated

proteins, the success of these studies are limited by HIV purification techniques that often result in co-purification of contaminating microvesicles.[102,103] HIV virions are small, dense particles of approximately 100 nm in diameter; HIV-infected cells produce microvesicles of similar size and density to that of the HIV virion, which have also been shown to share many of the components of HIV.[58] A variety of techniques have been employed to reduce microvesicle contamination, including CD45 depletion of microvesicles and affinity purification using viral envelope proteins.[104] Due to the large quantities of virions needed, affinity purification of virions or depletion of microvesicles is not a practical option for many biochemical studies. Therefore, we developed a novel strategy for purifying large quantities of HIV-1. By using cholesterol to manipulate the density of the particles in HIV-1 preparations (density modification; DM), we were able to separate virions from contaminating vesicles by centrifugation, making them suitable for analysis by tandem MS. Using this strategy, we were able to characterize host protein incorporation in HIV particles derived from CD4+ T cell lines. We compared our DM purification method with an orthogonal purification approach using inoxidol (OptiPrep) gradients by quantitative proteomics. Lastly, we tested our hypothesis that conserved proteins would reveal critical shared pathways by comparing our dataset of T cell derived HIV-1 virions to a reprocessed dataset of monocyte-derived macrophages (MDM) derived HIV-1 using the same bioinformatics pipeline.

We identified clusters of conserved proteins between MDM and T-cell derived HIV-1. These clusters included an extensive collection of actin isoforms and other core interacting proteins, many of which have previously been documented to interact with

viral proteins.[105,106] These data suggest that a limited number of viral-host protein interactions can explain the phenotypic diversity of HIV-1 virions produced from MDM or T-cells allowing HIV-1 to be incredibly plastic and opportunistic in its final protein composition depending on the cell type it is produced from. The common incorporation of syntenin-1 (a component of tetraspanin enriched membranes, TEMs) and CD44 (hyaluronic acid receptor) is suggestive of a common cellular egress pathway involving TEMs and a common vesicle population that targets hyaluronic acid enriched microenvironments.

Materials and Methods

Apparatus. MALDI-MS and MS/MS spectrum were obtained using an ABI 5800 MALDI TOF/TOF analyzer (AB Sciex) using a 2KeV extraction method with CID turned off using dynamic exit.

Reagents. $HIV_{MNCl.4}$ from either H9 (T-cell line) or CEMx174 (B-cell/T-¬cell hybrid line) cells was obtained from the AIDS and Cancer Vaccine Program (SAIC-Frederick).

Virus purifications. DM purification was accomplished by incubating virus in 420 μg/ml of cholesterol and 20 mM 2-hydroxy-beta cyclodextrin (βCD) in TNE as indicated, filtration through a 5 um filter on ice and pelleted through 20% sucrose for 1h at 100K x g. OptiPrep purification was performed as previously described.[107] SDS page, western blotting and EM were performed as described.[55]

Quantitative MS. Virus (normalized by p24) was ultracentrifuged and resuspended in 0.5 M triethylammonium bicarbonate with 1% rapigest, reduced (TCEP) and alkylated (MMTS) and subjected to tryptic digestion as previously described.[108] Peptides were labeled with iTRAQ reagents as follows for HIV-1 derived from CEMx174 cells: *m/z* 113: Control, *m/z* 114: DMP, *m/z* 115: OptiPrep, and for HIV-1 derived from H9 cells: *m/z* 117: Control, *m/z* 118: DMP, *m/z* 115: OptiPrep. Peptides were then subjected to nano rHPLC on a TEMPO-LC MALDI spotting system using a 90 minute gradient from 5% to 80% B (98 % ACN, 0.1 % TFA) at 500 nl min^{-1}, Matrix (CHCA, 5 mg / mL in 75 % ACN) was then supplemented to the flow post-column at 500 nl min^{-1}, and samples

were deposited onto a stainless steel plate at 10 second intervals. 1000 MALDI-MS and up to 1500 MS/MS spectrum were obtained using an ABI 5800 MALDI TOF/TOF analyzer (AB Sciex) using a 2KeV extraction method with CID turned off using dynamic exit. Spectral quality settings were set to high for both the spectrum and the iTRAQ reporter regions, according to manufacturer's suggestions (AB Sciex). ProteinPilot 3.0 was used to search UniProt-SwissProt with contaminants appended (2007.01.23; 254,765 sequences) with peptide threshold of 99.9%, and fixed modifications of iTRAQ (K, N-term), MMTS (C). Due to variable processing of gag, we subsequently normalized virus from different treatments and lines using cyclophilin A, a known gag interacting host protein[64] using the iTRAQ reporter bias correction feature built into ProteinPilot.

Tandem mass spectrometry (LCQ). 500 μg of capsid equivalents of DM purified HIV-$1_{MNCl.4}$ /H9virus was desalted and subjected to reverse phase HPLC analysis on a Beckman PF2D system as previously described into 37 fractions, digested and tandem MS performed as described.[109] Briefly, ESI-MS/MS of tryptic peptides was performed on an LCQ-ion trap-MS/MS instrument using a 60 minute gradient as previously described.[110] Data were acquired using Xcalibur 2.07 (Thermo, San Jose, CA). The three most intense ions (minimum signal of 100,000 ions) were selected for MS/MS fragmentation using a normalized collision energy of 35. Dynamic exclusion was applied for 30 seconds after 1 MS/MS acquisition, with a mass window of 2 Da.

Comparison between MDM derived virus and T cell HIV-1. 17 raw files for the analysis of MDM derived virus from a study published by Chertova et al.[74] were obtained from

the authors and analyzed in parallel with 37 DM purified fractions of HIV-1/H9. Briefly, peaks were selected and de-isotoped using DeconMSn for MDM derived LTQ data and using ReAdW (2009v) for LCQ derived HIV-1/H9. The data were then searched using PepArML[111], which uses multiple different search algorithms (OMSSA, X!Tandem with native, k-score and s-score scoring, MASCOT, MyriMatch, and InSpecT) as previously described.[112] Carbamidomethylation was set as a fixed modification and oxidized methionine was set as variable modification. Mass tolerances on precursor and fragment ions were set at 1.5, and .8 Da, respectively and missed cleavage as 1 using a specific search. The database used for search was the UniProt-SwissProt database (version 2010.11.02; 522,019 sequences). Peptides were combined on PepARML using a random forest approach (Weka)[113] and the results were then parsed into MASPECTRAS 2.0[114] with minimum 2 peptide for a protein and a spectrum false discovery rate of 5%. Peptides assigned to keratin were excluded, and since the analysis was focused on host proteins, viral peptide assignments were excluded. Protein redundancy was removed by MS based evidence clustering.[114] The data analysis pipeline meets all MIAPE standards[115] and the proteomics data have been deposited in the ProteomeExchange via the PRotein IDEntifications database (PRIDE) partner repository with the dataset identifier PXD000064.[116]

HIV protein interactions and network/pathway analysis. Network and pathways analysis was performed using the GeneMANIA gene network tool, which contains 353 human interaction networks based on data from BIND, IntAct and other interaction databases, using association data from protein and genetic interactions, known and predicted

pathways, co-expression, co-localization and protein domain similarity.[117] Our analysis was performed using only protein physical interaction data from GeneMANIA with GeneMANIA Cytoscape plugin. Identified host proteins were searched in the HIV-1, Human Protein Interaction Database for reported interactions in the literature.[118-120]

Safety considerations. All work with infectious HIV was performed in a biosafety level 3 environment using standard safety practices.

Results

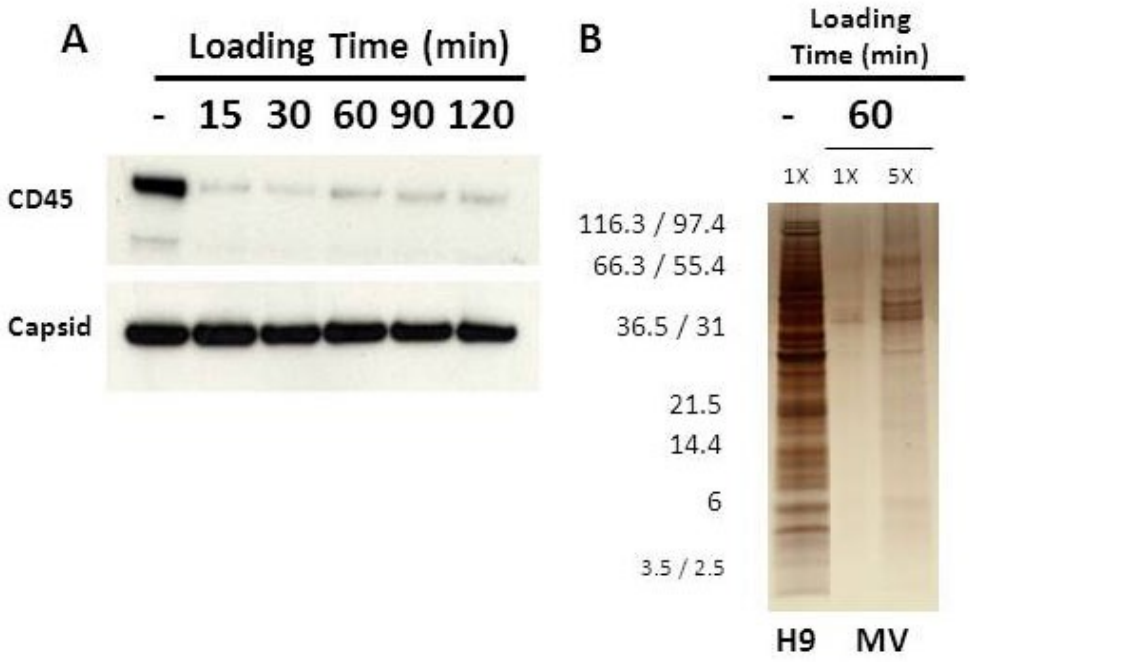
DM virion preparations allows for the separation of virus from microvesicles. The DM purification method is based upon our previous studies using beta-cyclodextrin to manipulate cholesterol in HIV-1 particles.[54,55] As viral purification through a 20% sucrose gradient results in co-purification of HIV and microvesicles of similar density, we differentially modified microvesicle and virion density by adding excess cholesterol to purified viral stocks. Cholesterol is differentially incorporated into microvesicles and virions, resulting in density changes that allow for the separation of highly purified virions from microvesicle contaminants. DM HIV-1 purity was assessed using CD45, a well-defined marker of vesicle contamination, resulting in a >90% reduction of material and elimination of microvesicles (Figure 3-1, A-C). DM HIV-1 had a <1 log infectivity decrease (not shown) and virion morphology was substantially altered (Figure 3-1,D).

To rule out artifact, we validated DM purified samples with an alternative purification method (OptiPrep; 60% iodixanol).[107] The relative abundances of proteins by quantitative proteomics (iTRAQ) from both samples were compared against virus pelleted through a 20% sucrose gradient. Virus preparations were carefully normalized by capsid protein (p24) by ELISA, and subsequently validated by SDS-PAGE (data not shown) prior to digestion with trypsin and labeling with iTRAQ reagents.

In an iTRAQ experiment, post-hoc corrections for the relative abundance of reporter ions are made to ensure that no single reporter is over-represented in the data analysis.[121] This can occur for a multitude of reasons, including variations in manufacturing of reagents or sample preparation conditions during labeling. Recently Breitwieser and colleagues

Figure 3-1. Purification of HIV-1 virions using density modification.

DM HIV-1 purity was assessed using CD45, a marker of vesicle contamination (Fig 1A). Isolated microvesicles (MV) from the H9 (Fig 1B) or Hut78 cell line (Fig 1C) were eliminated with this method (>90% of material was lost). HIV-1 infectivity was unchanged (not shown), but virion morphology was substantially altered (Fig 1 D, E).


A
Loading Time (min)
- 15 30 60 90 120
CD45
Capsid
B
Loading Time (min)
- 60
1X 1X 5X
116.3 / 97.4
66.3 / 55.4
36.5 / 31
21.5
14.4
6
3.5 / 2.5
H9 MV
C
Loading Time (min)
- 60
1X 1X
116.3 / 97.4
66.3 / 55.4
36.5 / 31
21.5
14.4
6
3.5 / 2.5
Hut78 MV
D
Control
E
Plus Cholesterol

showed that iTRAQ reporter intensities are valid over one log of dynamic range.[121] In this study, we manually adjusted iTRAQ reporter bias to ensure that no purification method resulted in a reporter ratio >1 when compared to the control preparation, as protein can only be depleted in the purification process.

For comparisons between viruses (CEMx174 vs H9 derived HIV-1), we initially attempted to adjust iTRAQ reporter bias based upon spectra assigned to HIV-1 capsid protein (p24). However, p24 resulted in unreliable bias estimation secondary to the extreme sequence divergence of HIV capsid protein and improperly assigned viral peptides by ProteinPilot. Instead, we investigated the use of spectra assigned in control preparations to the host protein cyclophilin A (CypA). The host protein CypA has been reported to be included in virions,[64] but has not been reported to be present in microvesicles except under conditions of extreme cellular stress, such as cellular irradiation.[122,123] CypA incorporation has also been reported to be important for maximal HIV infectivity and it has been suggested that the absence of CypA incorporation leads to HIV restriction.[65] HIV recruits CypA to ~10% of its capsid monomers in newly assembled cores and the CypA binding site on capsid is highly conserved in all primate lentiviruses.[64,65] We therefore adjusted iTRAQ reporter bias using CypA peptides, whilst ensuring that the most abundant protein was normalized to a 1:1:1 ratio between different preparations. Indeed, this method did not violate our rule of host proteins in the purification groups being less than control. Final adjustments were minor and accounted for a 25% decrease in CypA for CEMx174 derived HIV-1 and a 35% decrease in CypA for H9 derived HIV-1 using DM purification. Comparatively, a 10% and 40% decrease in

CypA were observed using OptiPrep purification for CEMx174 and H9 derived HIV-1, respectively. These results suggest that CypA may indeed be present in microvesicles induced by HIV-1 infection, like other forms of cellular stress.

For virions produced in either H9 or CEMx174 cells, we observed a decrease in protein abundance for both DM and OptiPrep purified virions compared to control methods. DM purification significantly reduced the abundance of 34 proteins for CEMx174-derived HIV-1 virions, whereas OptiPrep purification resulted in significant reductions of 8 proteins (Table 3-1). Similar results were observed for H9 cells (data not shown). Many proteins that were reduced in quantity for either DM or OptiPrep purification have been shown to be in microvesicles;[124-126] the greater reduction in proteins using DM purification suggests that this method is a more stringent purification measure than OptiPrep purification. However, many of the reduced proteins have also been shown to be incorporated into HIV virions and we cannot rule out the loss of a subset of viral particles in either purification method.[58]

iTRAQ analyses can differentiate HIV-1 virions derived from a T cell line and a B cell/T cell hybrid cell line. Viral stocks produced from different cell lines displayed unique phenotypes, and virion composition reflected the progenitor cell type. DM purified viral stocks derived from CEMx174 and H9 cells were compared. Table 2-2 shows that 15 proteins can be used to differentiate between the cell lines. Notably, virions produced from CEMx174, which is a T-cell/B-cell hybrid,[127] contained higher levels of CD48 antigen precursor, a marker of B-cell activation.[128]

Table 3-1. Reduced proteins in HIV-1 derived from CEMX174 cells following virion purification using DM or Optiprep.

% of Sequence Coverage	Accession Number	Protein Name	Peptides (95%)	DM: Control	*P-val*	Optiprep: Control	*P-val*
42.9	P63104	14-3-3 protein zeta/delta	6	0.5151	*0.0371*	0.7533	0.0613
56.5	P63261	Actin, cytoplasmic 2	29	0.5099	*0.0013*	0.92	0.1083
41	P06733	Alpha-enolase Homo sapiens (Human)	9	0.5058	*0.0001*	0.7502	*0.0038*
33.3	P07355	Annexin A2	9	0.4459	*0.0015*	0.8047	*0.0188*
61.2	P80723	Brain acid soluble protein 1	7	0.523	*0.0409*	0.7312	*0.0264*
23.5	P09326	CD48 antigen precursor (B-lymphocyte activation marker BLAST-1)	5	0.4887	*0.005*	0.949	0.5258
41.5	O00299	Chloride intracellular channel protein 1	5	0.5008	*0.0012*	1.0572	0.5918
28	P31146	Coronin-1A	4	0.5112	*0.0003*	0.8208	*0.011*
30.1	P68104	Elongation factor 1-alpha 1	8	0.5466	*0.0006*	0.9394	*0.4253*
50	P15311	Ezrin (p81)	12	0.5889	*<0.0001*	0.8771	0.0974
26.3	P04406	Glyceraldehyde-3-phosphate dehydrogenase	3	0.4113	*0.0231*	0.8528	0.5458
51.2	P11142	Heat shock cognate 71 kDa protein	18	0.5481	*0.0042*	0.9216	0.5634
29.1	P08238	Heat shock protein HSP 90-beta	8	0.5316	*0.003*	1.0241	0.8397
46.6	P30453	HLA class I histocompatibility antigen, A-34 alpha chain precursor	14	0.6886	*0.0117*	0.9655	0.7305
31.1	P01903	HLA class II histocompatibility antigen, DR alpha chain precursor	7	0.4474	*0.007*	0.9419	0.4304
52.6	P13760	HLA class II histocompatibility antigen, DRB1-4 beta chain precursor	7	0.5259	*0.0458*	0.9599	0.7407
41.7	P13761	HLA class II histocompatibility antigen, DRB1-7 beta chain precursor	7	0.5595	*0.001*	0.9605	0.6705
25.9	P05362	Intercellular adhesion molecule 1 precursor	3	0.5685	*0.0489*	0.8179	0.1331
57.5	P26038	Moesin	19	0.5428	*0.0012*	0.9082	0.0732
18.4	Q09666	Neuroblast differentiation-associated protein AHNAK	2	0.4589	*0.0143*	0.7738	*0.025*
19.2	P43007	Neutral amino acid transporter A	5	0.3368	*0.0098*	0.8818	0.5752

32.2	Q06830	Peroxiredoxin-1	5	0.3815	*0.0157*	0.7814	*0.0282*
23	P13796	Plastin-2 (L-plastin)	4	0.5221	*0.0042*	0.8782	0.1899
16.4	Q8WUM4	Programmed cell death 6-interacting protein	2	0.5772	*0.0397*	0.8987	0.5737
16.2	P30101	Protein disulfide-isomerase A3 precursor	1	0.4702	*0.0423*	0.7946	0.3432
23.4	P14618	Pyruvate kinase isozymes M1/M2	5	0.5098	*>0.0001*	0.926	0.2772
26.6	P15153	Ras-related C3 botulinum toxin substrate 2 precursor	2	0.5057	*0.0433*	0.963	0.811
24.5	P62834	Ras-related protein Rap-1A precursor	1	0.4817	*0.0063*	0.7928	*0.0465*
81.8	P62328	Thymosin beta-4	3	0.4805	*0.0215*	1.0323	0.7312
31.6	P61586	Transforming protein RhoA precursor	2	0.4166	*0.0123*	0.9393	0.5786
37.1	P67936	Tropomyosin alpha-4 chain	3	0.506	*0.0248*	0.8225	0.3176
17.6	Q9BQE3	Tubulin alpha-6 chain	3	0.4275	*0.0074*	0.8795	0.2247
72.4	P62988	Ubiquitin	3	0.4191	*0.0037*	0.9396	0.5418

Table 3-2. Proteins able to differentiate between HIV-1 derived from CEMX174 cells or H9 cells.

% of Sequence Coverage	Accession Number	Protein Name	Peptides (95%)	CEM:H9	P value*
61.2	P80723	Brain acid soluble protein 1	7	6.1243	0.042
23.5	P09326	CD48 antigen precursor	5	1.8935	0.0377
45.8	P23528	Cofilin-1	6	2.1352	0.0266
29.4	P04075	Fructose-bisphosphate aldolase A	4	2.2855	0.0409
33	P04899	Guanine nucleotide-binding protein G(i), alpha-2 subunit	5	2.3068	0.0296
46.6	P30453	MHC class I antigen A*34	14	3.3001	0.0184
16.9	P04233	HLA class II histocompatibility antigen gamma chain	2	4.2203	0.0346
47.9	P07737	Profilin-1	4	2.4426	0.0169
23.4	P14618	Pyruvate kinase isozymes M1/M2	5	1.8639	0.016
26.4	Q15286	Ras-related protein Rab-35	3	2.054	0.013
21	P05023	Sodium/potassium-transporting ATPase alpha-1 chain precursor	7	2.0799	0.0344
81.8	P62328	Thymosin beta-4	3	2.4172	0.0273
37.1	P67936	Tropomyosin alpha-4 chain	3	2.7548	0.0016
17.6	Q9BQE3	Tubulin alpha-6 chain	3	1.9302	0.0058
19.1	P07437	Tubulin beta chain	3	1.6285	0.0268

*P<0.05 cut-off.

Shotgun analysis of DM modified HIV-1 identifies 283 host proteins. While our iTRAQ experiments provided us with a powerful method of determining our relative purification efficiency, no multidimensional protein separation strategies were used for this experiment. Therefore, to extend our coverage of the DM HIV proteome, we performed HPLC separation of DM-HIV-1, and collected 37 fractions that were then subject to analysis by MS/MS on LCQ-duo equipped with an Agilent nano-HPLC system. The resulting spectra were pooled and searched on our MS-analysis pipeline. We found that the peptides were assigned to >1800 proteins, including redundant assignments; these proteins clustered to 283 individual host proteins using a spectrum false discovery rate of 5%.

MDM and T cell-derived HIV-1 virions incorporate a limited number of shared host proteins. To determine which host proteins were incorporated to the virion both in T- and in macrophage-cell types, we compared our T cell dataset to the MDM-derived HIV-1 dataset generated by Chertova and colleagues.[74] To ensure that the datasets were comparable, the Chertova dataset was reanalyzed using our data analysis pipeline.[129] 136 (38 clustered) proteins were unique to the macrophage derived virus, and 1339 (241 clustered) proteins were unique to the T-cell derived virus. 680 (79 clustered) proteins were shared between the MDM derived and T-cell derived dataset (Figure 3-2). Of the 79 common proteins, many of these proteins were isoforms of the same protein with different peptides identified (Table 3-3). The majority of these proteins were distinct actin isoforms. Other proteins of note were ERM proteins, the dynamin domain containing

Figure 3-2. Comparison between the published MDM derived HIV-1 and other datasets. (A) Comparison of the published MDM-derived HIV-1 dataset by Chertova et al. and the dataset after reanalysis through our bioinformatics pipeline. Based on matching protein accession numbers, 42 proteins from the reanalysis are common to the published list. (B) Comparison of the published MDM-derived HIV-1 dataset by Chertova et al. with the T cell-derived HIV-1 dataset. The two datasets contain 35 proteins in common. (C) Comparison between the reanalyzed MDM derived HIV-1 dataset and the T cell-derived HIV-1 dataset. 76 clusters of proteins are in common to both cell types. *clustered proteins; **total number of proteins identified from the data set.

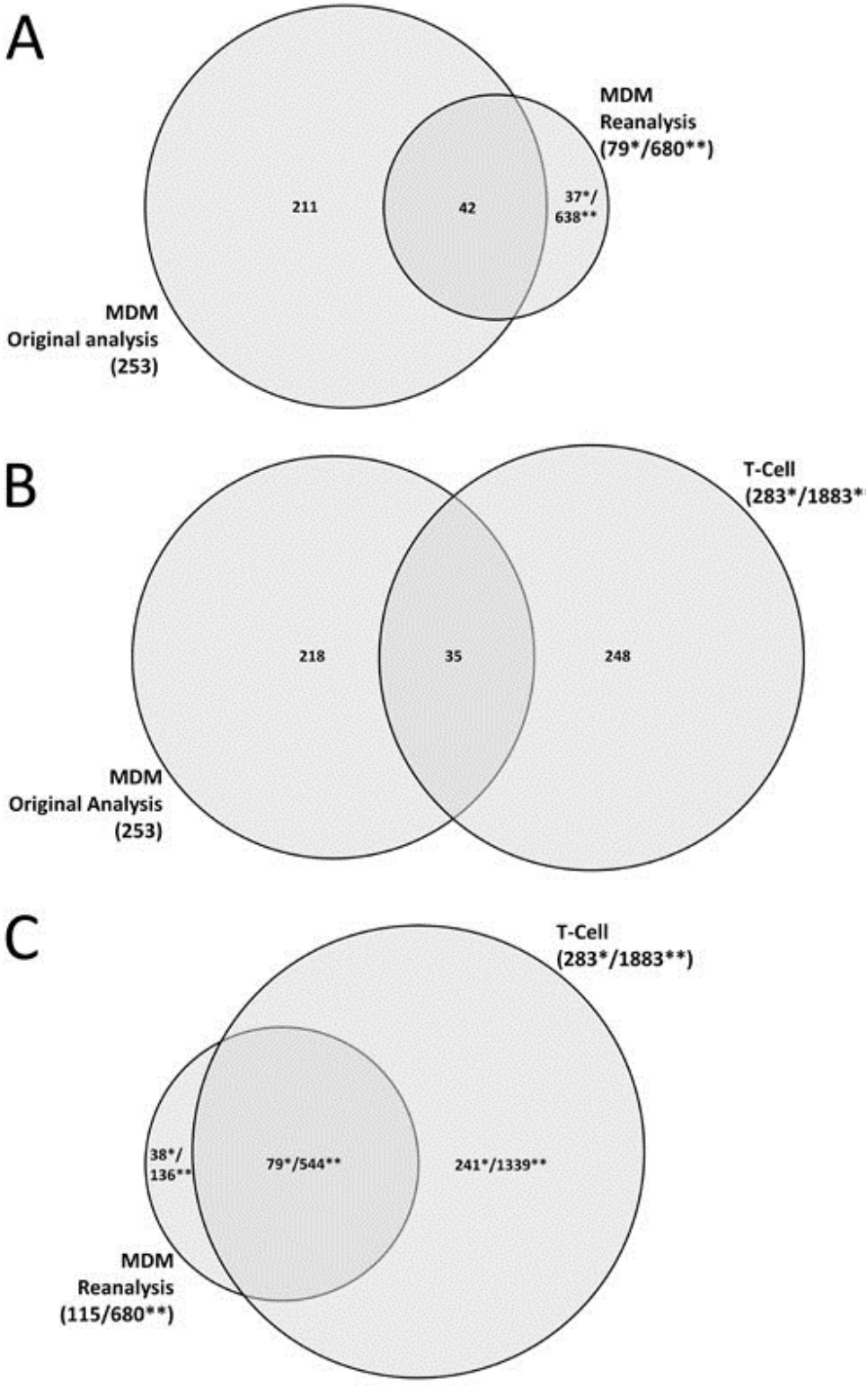

A
MDM
Reanalysis
(79*/680**)
211
42
37*/
638**
MDM
Original analysis
(253)
B
T-Cell
(283*/1883*
218
35
248
MDM
Original Analysis
(253)
C
T-Cell
(283*/1883**)
38*/
136**
79*/544**
241*/1339**
MDM
Reanalysis
(115/680**)

Table 3-3. Identified proteins common to MDM- and T-cell derived HIV-1 virions.

Gene Name	Gene Symbol	% of Sequence Coverage	No. of Unique Peptides	No. of Unique Spectra
2',3'-cyclic-nucleotide 3'-phosphodiesterase	CNP	11.17	5	7
6-phosphogluconate dehydrogenase, decarboxylating	Pgd	9.94	3	5
Actin (37 isoforms)	act1	12	827	8432
Alpha-1-antiproteinase	SERPINA1	32.22	17	38
Alpha-2-H	AHSG	25.35	9	16
Annexin A2	ANXA2	52.22	21	62
CD44 antigen	CD44	7.28	4	14
Cell division control protein 42 homolog	CDC42	36.65	6	26
EH domain-containing protein 4	EHD4	24.59	9	12
Elongation factor 1-alpha (4 isoforms)	eFF1a	10.9	6	78
Ezrin	EZR	18.44	9	23
Heat shock 70 kDa protein (11 isoforms)	HSPA	15.78	132	516
Heat shock cognate 71 kDa protein	HSPA8	39.63	26	126
Heat shock protein H	HSP90AB1	24.45	15	52
Hemoglobin fetal subunit beta	-	60.69	10	101
Hemoglobin subunit beta (3 isoforms)	HBB	27.9	4	18
HLA A (MHC class I)	HLA-C	19.13	10	39
HLA DR (MHC class II) (3 isoforms)	HLA-DRB1	21.06	5	28
Moesin	MSN	23.4	15	70
P (Ankyrin Repeat Containing)	POTEE	10.33	16	168
Peptidyl-prolyl cis-trans isomerase A (cyclophilin A)	PPIA	56.37	14	103
Phosphoglycerate kinase 1	PGK1	32.86	12	22
Pyruvate kinase isozymes M1/M2	PKM2	40.12	16	40
Ras-related C3 botulinum toxin substrate 2	Rac2	30.73	6	7
Syntenin-1	SDCBP	10.2	7	9
Ubiquitin (Fragment)	-	39.69	4	27

protein EH4, a phosphodiesterase, CypA and heat shock proteins. The only conserved membrane proteins identified were syntenin-1 (a TEM protein) and CD44 (hyularonic acid receptor), a marker presently used in commercial kits to enrich HIV.

As the conserved set of proteins may represent important cellular partners for the HIV virion, we conducted a literature search to determine whether the identified host proteins have been reported to be relevant in the HIV lifecycle and interact with viral proteins (Table 2-4). Out of the 26 protein clusters reported, 16 have previously been described in association with HIV-1, and 10 represent previously undefined associations.

To determine if the conserved set of proteins between MDM and T-cell derived HIV-1 could be used to reconstruct the protein composition of each virion, we seeded the GeneMANIA human network database with the core set of proteins and allowed for the 1000 most-related interacting partners. We found that 29% and 53% of host proteins from T cell derived or MDM derived HIV-1, respectively, could be explained by primary interactions with the conserved set.

Table 3-4. HIV-1-human protein interactions reported in the literature.[118-120]

Host Protein	HIV Protein Interaction
2',3'-cyclic-nucleotide 3'-phosphodiesterase	Activated by: tat
Actin	Inhibited by (multiple isoforms): env Binds (multiple isoforms): gag, nef Interacts with (multiple isoforms): gag Associates with (multiple isoforms): nef rearrangement induced by (multiple isoforms): nef, tat Cleaves (multiple isoforms): pol Associates with (beta-actin): rev Downregulated by (multiple isoforms), upregulated by (beta actin): tat Polymerization enhanced by (gamma 1 propeptide): vpr
Alpha-1-antiproteinase	Interacts with: env
Annexin A2	Colocalizes with, interacts with (isoform 1): gag Downregulated by (isoform 2): tat
CD44	Downregulates: vpr
Cell division control protein 42	Inhibited by, interacts with, upregulated by: nef
Eukaryotic translation elongation factor 1 alpha 1	Inhibited by: gag Binds: gag, pol Interacts with: tat
Ezrin	Binds: env Interacts with: env, gag Incorporates: gag Upregulated by: vpr
heat shock protein 70kDa	Interacts with (protein 5), upregulated by (multiple proteins), inhibits (multiple proteins): env Incorporated by (multiple proteins), stimulates (multiple proteins), inhibits (protein 8): gag Regulates (multiple proteins): tat Inhibits (protein 1a), binds (protein 1a), competes with (multiple proteins): vpr
MHC Class I	Interacts with, complexes with: env Binds: env, gag, nef, pol Upregulated by: env, tat Colacalizes with, inhibited by, modulated by: nef Downregulated by: nef, tat, vpu
MHC Class II	Associates with, incorporated by: env Upregulated by: env, tat Inhibited by, interacts with: env, nef Colocalizes with, relocalizes, relocalized by: gag Downregulated by: gag, nef, tat
Moesin	Binds, relocalized by: env Incorporated by: gag
Peptidyl-prolyl cis-trans isomerase A (cyclophilin A)	Inhibited by, required by: env Incorporated by, modulates, interacts with, stabilized by: gag Binds: gag, nef, vif Isomerizes: gag, vpr
Ras-related C3 botulinum toxin substrate 2	Interacts with: nef Activated by, downregulated by: tat
Syntenin-1	Upregulated by: env
Ubiquitin	Ubiquinates: gag, rev, tat

No interactions have been reported for 6-phosphogluconate dehydrogenase, decarboxylating; Alpha-2-H; EH domain-containing protein 4; HSP90AB; Hemoglobin

fetal subunit; HBB; P (Ankyrin Repeat Containing); Phosphoglycerate kinase 1; Pyruvate kinase isozymes M1/M2

Discussion

Using a novel HIV purification assay we have found a common set of host proteins that are incorporated into virions produced from monocyte-derived macrophages (MDMs) and T cells. DM purification modifies the density of microvesicles, allowing for the purification of large quantities of microvesicle-free viral stocks. This method may not be necessary for virion purification from infected MDMs, since these cells have longer half-lives than T cells, allowing for higher virion yields, and MDMs produce a lower level of contaminating microvesicles compared to lymphocytes.[74,102,103] We found that DM clears >90% of microvesicles using CD45 as a marker protein (by densitometry, not shown). We compared this method to the OptiPrep (60% iodixanol) method for microvesicle-free HIV-1 purification.[107] Results were normalized by CypA, as CypA has been reported to be incorporated into viruses in an approximately 1:10 ratio to gag particles.[64,65] While using a viral protein for normalization might seem like an adequate normalization tool, viral protein sequence divergence and differential processing by progenitor cell type make it difficult to normalize by these proteins. Both OptiPrep and DM purification methods produce consistent results, reducing levels of proteins known to be incorporated in microvesicles. DM purification proved to be a more stringent approach, as there was a greater reduction in CypA levels coupled with a higher number of significantly reduced proteins compared to OptiPrep methods. Ott and colleagues developed a purification technique based on similar principles, in which proteins in microvesicles are digested with the nonspecific serine protease subtilisin.[105] The subtilisin digestion decreases microvesicle density, allowing for purification of HIV particles by density gradients, allowing for >95% purification of virions.[104] Subtilisin treatment digests membrane

proteins, though, and is only suitable for determining the composition of proteins inside the virion.[105]

We cannot rule out that DM purification also modifies viral composition in some manner, as cholesterol has been reported to be an integral component of the viral membrane, and that this may account for some of the protein reduction.[54,55] Notably, electron micrographs of DM-treated virions show some membrane irregularities, which could impact protein composition of the purified viral stocks. This may explain the absence of some host membrane proteins which have been reported to be incorporated into HIV virions. Of note, tetraspanin proteins were not detected in our analysis. This may not be surprising, though, given that tetraspanin interactions are affected by cholesterol and the DM assay may have disrupted TEMs.[130] It is of note that very few membrane-bound proteins were observed in the common set of proteins, particularly given that TEMs have been shown to be of importance in HIV-1 biology.[131-133] We did detect the PDZ-containing protein syntenin-1 in both MDM- and T cell-derived virions. Syntenin-1 has been shown to have a large variety of interaction partners, including syndecan, Rab 5, Rab 7, CD63, and phosphoinositol lipids. Many of the partners for syntenin-1 are involved in membrane trafficking, including tetraspanin and TEM-associated proteins.[134,135] Since TEM components are frequently reported in the viral envelope, but were not detected in this analysis, it is possible that syntenin-1 is involved in the HIV-1-TEM interaction, and that the syntenin-1-TEM interactions were disrupted by our purification process. This may implicate syntenin-1 as an important mediator of viral envelope composition. However, preliminary data with siRNA knockdown of syntenin-1

in HIV-infected Jurkat cells has not demonstrated any effect on virion production (data not shown), so the importance of syntenin-1 in the HIV lifestyle remains speculative.

The incorporation of CD44, a receptor for hyaluronic acid, also provides potential fitness benefits for a lentivirus. HIV replicates in activated T cells, so an attachment to hyaluronic acid may allow the virus to target areas of inflammation, as CD44 induction is a first step of immune activation and also involved in T cell trafficking.[136] Notably, it has been reported that CD44 cell-surface expression is lost in HIV-infected monocytic cell lines, resulting in cell aggregation.[137]

Using quantitative proteomic analyses on DM-purified input, we were able to differentiate between viral stocks prepared from the H9 T cell line and the CEMx174 B cell/T cell hybrid line. There were several proteins which could differentiate between the two stocks, including the B cell activation marker CD48 precursor, which is not unexpected considering the cell line origins[128]. These results indicate that proteomic analysis in combination with several purification techniques can be used to differentiate viral stocks from multiple sources. These methods may be applicable to identifying the cell source of virus produced from latently infected cells. These methods may also be used to differentiate between viruses from various cellular reservoirs within the host.

We further compared DM purified viral stocks from H9 cells to a published database of MDM-derived viral stocks. To increase the validity of this comparison, raw data from the Chertova study collected using similar MS instrumentation employed in the current study

was reanalyzed using the bioinformatics pipeline developed by our group. This method ensured that both datasets were analyzed using the same stringent search criteria. Using this comparison, we identified a common set of 26 proteins that are incorporated into HIV virions produced in both MDM and T-cell lines. As these proteins are incorporated in virions produced in both cell types, we hypothesize that these proteins may have direct interactions with viral proteins or may be important in the viral life cycle.

The base host protein composition in virions is mainly actin, chaperones, CypA and a handful of other proteins. Many of these have been shown to have functional impact on the HIV lifecycle. The importance of CypA on the viral life cycle has been well documented.[138] CypA is believed to regulate the capsid interaction with host factors, either during uncoating or during other lifecycle processes.[139] The protease α1-antitrypsin (SERPINA1; AAT) blocks protease cleavage of gp160 and gag polyprotein. Thus, it is not surprising that HIV protease binds and cleaves AAT, and this binding may account for the incorporation of AAT in HIV particles.[140-142] Our results suggest that HIV-1 virions have evolved to target a pathway enriched in ERM family proteins and vesicle trafficking, based on the conserved incorporation of EHD4 in T cell and MDM derived virions. EHD4 has been shown to regulate transport from the early endosome to the recycling endosome and the late endocytic pathway.[143,144] EH domains bind Rab proteins, which are known to interact with the HIV protein rev.[145] A recent study has suggested a role of HSP90AB in HIV replication. Inhibition of HSP90AB resulted in anti-HIV activity *in vitro*, with ritonavir-resistant viruses showing hypersensitivity to the inhibitor.[146] Another agent with anti-HIV activity was found to bind HSP90AB and

prevent dimerization.[147] Annexin A2, a protein involved in membrane trafficking, likely bridges the gap between the cytoskeletal proteins and the viral membrane. Annexin A2 binds HIV gag and siRNA knockdown of the protein can reduce infectivity of virions generated in MDMs, but this may be cell type dependent.[148-150]

Other identified proteins, including cytoskeletal and HLA proteins have repeatedly been reported in the literature as interacting with HIV proteins.[69,74,105,151] Further, many of the proteins identified in this study, including actin, 2',3'-cyclic-nucleotide 3'-phosphodiesterase, CypA, EEF1A-1, ezrin, annexin 2, HSP70, and HSC71, have also been identified in a quantitative proteomic analysis of an HIV-1 lentivirus vector produced in 293T cells.[152] Thus, this common set of proteins identified in this study is recapitulated by findings by other groups.

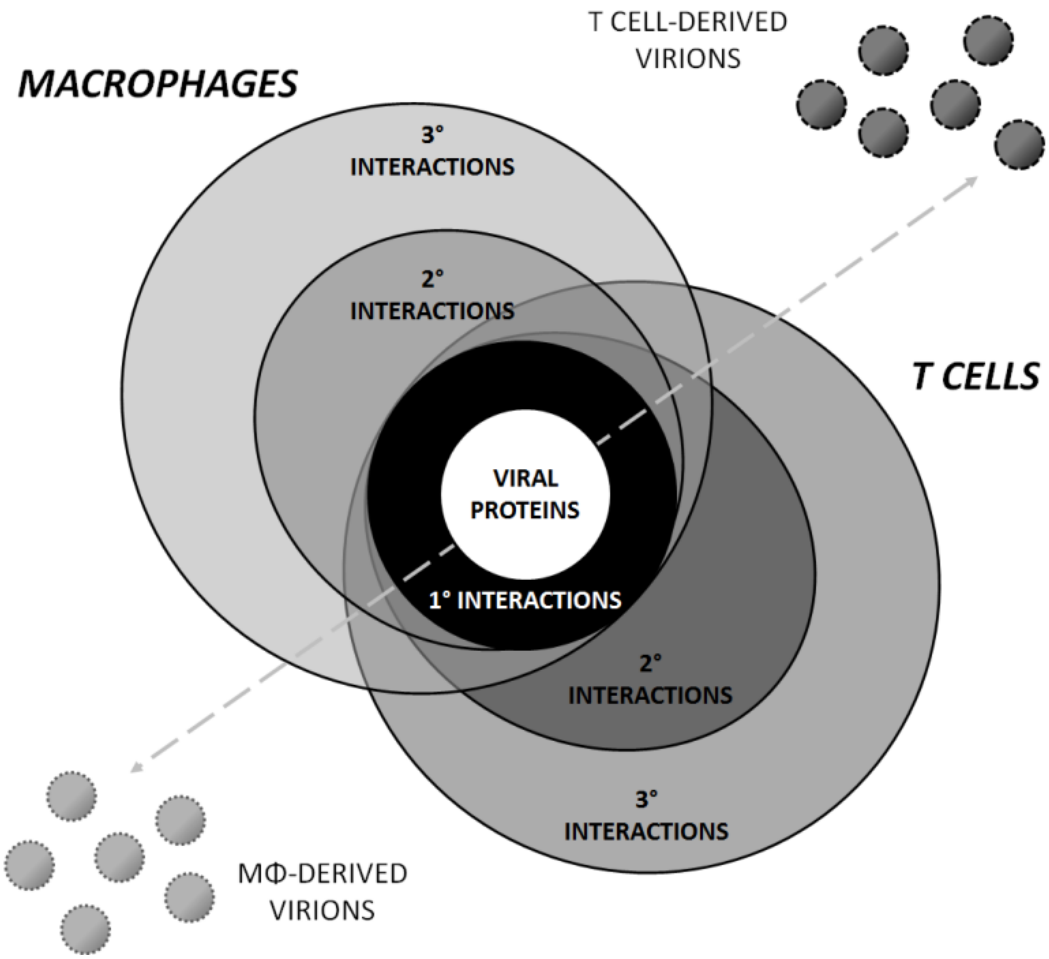
Given the large number of host proteins incorporated into the virus and the limited number of viral proteins, it is plausible that only a few specific interactions between virus and host proteins allows it to package a large array of host proteins. Host proteins that have a direct interaction with HIV proteins would serve as protein hubs. The 26 proteins identified to be common to the two different cell types are predicted to interact with >1000 related human proteins. Many of these secondary interacting proteins are commonly reported to be incorporated into virions, including ERM proteins and adhesion molecules. By assigning query based network weights (GeneMANIA, see methods), these associational proteins are predicted to interact with 62% and 38% of the proteins common to T cell and MDM derived HIV-1 respectively. However, it is important to note

that many more T-cell derived proteins were identified than MDM HIV-1 derived proteins, so this may skew this analysis. Additionally, protein prediction network algorithms are based on protein-protein interactions; other interactions (e.g., lipid or nucleic acid mediated) are not modeled. Thus, only a few direct interactions within the virus may dictate the host protein composition in nearly limitless dimensions. Ultimately, the host protein composition, as well as interaction differences between cell types, may drive virion phenotypic diversity, despite conserved viral protein-host protein interactions between cell types (Figure 3-3). While we do not intend to minimize the functional importance of other host proteins incorporated into HIV-1 outside of this minimal set of proteins, it is likely that therapeutic strategies targeting proteins other than these core proteins would result in limited efficacy due to the high degree of plasticity apparent with HIV. Therefore, we would propose that therapeutic or drug development efforts targeting host-virus interactions be focused on interacting proteins showing direct interaction with HIV proteins that are conserved between T cells and macrophages.

Finally, this study demonstrates the critical nature of harmonized data analysis when making inter-study protein comparisons. Existing studies have demonstrated the lab-to-lab and instrument-to-instrument variability in proteomics studies of identical samples, as well as search results from different search algorithms.[153,154] Our re-analysis of the historical data from Chertova *et al* using current FDR-driven statistical analysis resulted in a truncated list of virally incorporated host proteins, comparable to what we observed experimentally in our work. This demonstrates the need for archiving of instrument raw data files so they may be subject to reinterpretation as bioinformatics improvements are

Figure 3-3. Hypothetical schematic of the impact of primary HIV-host protein interactions on virion phenotype.

HIV proteins interact with a common set of host proteins that is found in multiple cell types capable of sustaining HIV infection. These common set proteins have secondary and tertiary interactions with both cell-specific and common protein partners and these interactions determine the phenotype of released virions. Thus, despite a limited number of HIV-host protein interactions, viral diversity is driven by the secondary and higher interactions based on cell-type.


T CELL-DERIVED
VIRIONS
MACROPHAGES
3°
INTERACTIONS
2°
INTERACTIONS
T CELLS
VIRAL
PROTEINS
1° INTERACTIONS
2°
INTERACTIONS
3°
INTERACTIONS
MΦ-DERIVED
VIRIONS

developed, and highlights the danger of making protein comparisons from tables in the published literature, particularly with regard older data that was not filtered by FDR or another stringent statistical measure.

Using a proteomic analysis approach, this study identifies proteins that are incorporated into the virus in multiple cell types, and many of these proteins have been shown to be relevant to the HIV life cycle. These proteins may represent important conserved interactions and, therefore, could be targets for interventional strategies.

CHAPTER IV

THE ROLE OF TETRASPANIN PROTEINS IN HOST PROTEIN INCORPORATION INTO THE HIV-1 ENVELOPE

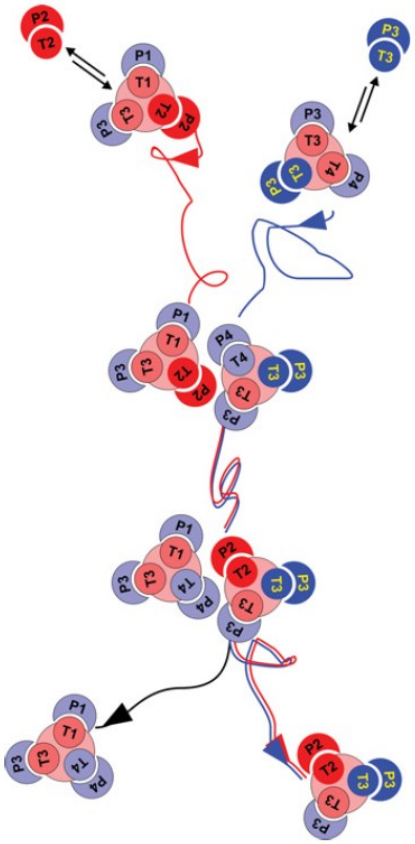
Introduction

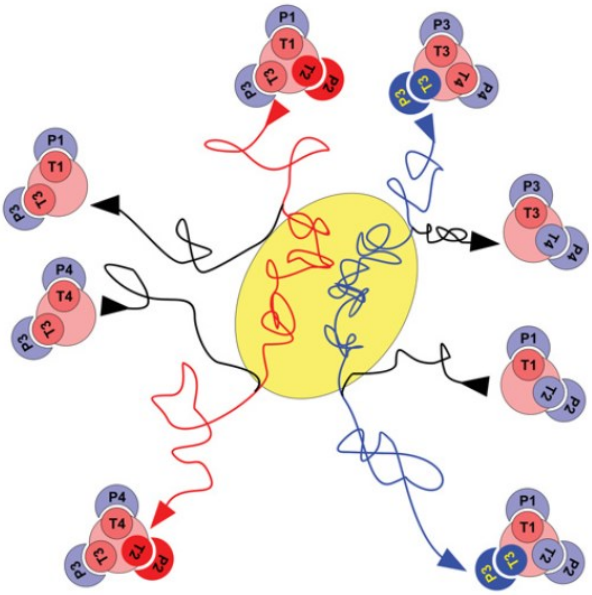
Tetraspanin family proteins are four-pass membrane proteins that are highly conserved across vertebrate organisms. Tetraspanin proteins have a small extracellular loop (SEL; 13-30 amino acids), large extracellular loop (LEL; 70-140 amino acids), short intracellular loop, and C- and N-terminal tails.[155] There are 33 known human tetraspanin proteins, with tetraspanin proteins found in virtually all cell types.[156] Experimental data have shown tetraspanin family members to be involved in cell fusion,[157,158] adhesion,[159-166] cell spreading,[167] proliferation, signaling,[168-170] intracellular vesicle trafficking,[171-173] bacterial and viral lifecycle,[174-184] immune response,[169,185-190] vascular morphogenesis and remodeling,[159,160,162,191] tumor progression and metastasis,[192,193] neurite navigation, thrombosis, and apoptosis.[194] Due to the large number and diversity of functions, tetraspanins have been hypothesized to ultimately function as membrane organizers. [reviewed by Martin Hemler[195,196]]

As tetraspanins have been reported to be important in many different cellular functions, they must be capable of directly or indirectly interacting with a large number of proteins, including cytoskeletal proteins, adhesion molecules, proteins involved in the immune response, and many other proteins with varying functions.[197,198] Many of these interactions have been shown to be mediated through the LEL.[161,169] Tetraspanins and their interacting proteins form what is often called tetraspanin enriched microdomains (TEMs). TEMs can be viewed as a clustered microdomain, in which tetraspanins and their partners fluidly interact with frequent contact exchanges, as illustrated in Figure 4-1 [reproduced from Charrin 2009].[199] This is supported by single molecule interaction

Figure 4-1. A dynamic view of the tetraspanin web.[199]

This model is based on biochemical analysis of the tetraspanin web and the recent analysis of the dynamic of CD9 and CD151. For clarity, two tetraspanin/partner pairs are labeled in red and blue, and the traces correspond to their movement. Left: basal level of interactions: small clusters of tetraspanins (T1, T2, . . .), each specifically associated with a molecular partner (P1, P2, . . .), would patrol in the plasma membrane, frequently interacting with other clusters and exchanging some of their constituents, contributing to the diversity of interactions within the tetraspanin web. Right: upon particular stimulations, which remain largely to be defined, some tetraspanins become confined within discrete areas of the plasma membrane where more stable interactions take place. The model shown here is based on the transfer of one tetraspanin to the tetraspanin-enriched areas, but other models such as the gathering of several clusters are possible.[199] [This research was originally published in *Biochemical Journal*. Charrin S, le Naour F, Silvie O, Milhiet PE, Boucheix C, Rubinstein E. Lateral organization of membrane proteins: tetraspanins spin their web. *Biochem J*. 2009;420:133-154. ©the Biochemical Society]





studies indicating that diffusional trapping through protein-protein interactions concentrate or exclude proteins from lipid ordered domains.[200] Thus, TEMs are believed to be dynamic microdomains that are enriched in specific proteins, although the protein composition of TEMs will likely vary depending on cell type and cell status. In this model, there is likely to be temporal variance in a single TEM as well.

In addition to being enriched in specific proteins, TEMs are also enriched in specific lipids, including sphingomyelin, glycosphingolipids and cholesterol.[201] Notably, the lipid composition of TEMs is similar to that of "lipid rafts" and, therefore, TEMs can be viewed as lipid ordered domains.[197,198] This has implications for HIV biology, as HIV has been shown to bud from lipid ordered domains. Further, many tetraspanins are localized to late endosomes/multivesicular bodies (MVBs).[125,202-214] This is also the site of HIV assembly in macrophages.[213,215] It is, therefore, not surprising that tetraspanins are frequently reported to be incorporated in the viral envelope.[74] Indeed, several studies have shown that TEMs serve as the budding site for HIV in macrophages and CD4+ T cells.[215-217] Data also indicate that tetraspanins and associated proteins are important in HIV envelope fusion, with one group indicating that tetraspanins are required for productive HIV infection in macrophages.[218-220] Others have shown that the extracellular domain of CD63 can inhibit HIV infection in macrophages.[221] Additionally, CD81 downregulation or blocking by antibody in MOLT cells decreased HIV release.[133] Surface CD81 expression is decreased in CD4+ T cells in HIV-infected persons, compared to healthy controls; however CD81 mRNA increases in these subjects, suggesting post-translational changes in CD81 protein.[222] As CD81 is incorporated into

HIV virions, it is conceivable that CD81 decreases on the surface of CD4+ T cells is due to viral budding.

It should also not be surprising that many of the proteins reported to be found in TEMs are also incorporated into the HIV envelope and that the HIV envelope is similar in lipid composition to TEMs. Based on these similarities, it is possible that tetraspanins serve as a primary determinant of host protein incorporation in HIV virions and, by extension, as the master regulators of HIV phenotype.

Materials and Methods

Cell culture. All cells were propagated in RPMI supplemented with 10% FBS (HyClone), 100 μg/ml streptomycin, and 100 U/ml penicillin.

Flow Cytometry. Flow cytometry was performed by first fixing cells with phosphate-buffered saline (PBS) containing 2% paraformaldehyde and then permeabilizing cells with PBS buffer containing 1% BSA, 5% normal goat serum (NGS), and 0.2% saponin, followed by staining with appropriate antibodies (Abs). Infection of cells was determined by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated anti-p24 (Coulter). The primary MAbs were detected with Alexa Fluor 647-conjugated goat anti-mouse polyclonal antibody. Virus production was measured by anti-p24gag enzyme-linked immunosorbent assay (ELISA). Compensation was performed with single-stained cells. Data were collected on FACSCalibur (BD Biosciences) instruments and analyzed with FACSDiva (BD Biosciences) or Flowjo (Tree Star) software. For immunofluorescence staining, cells were fixed with 2% paraformaldehyde in PBS and permeabilized with PBS containing 5% NGS, 1% BSA, and 0.25% Triton X-100. Cells were than stained with indicated primary and secondary Abs.

Transfections. Transient transfections were performed using Lipofectamine 2000 (Invitrogen). The siRNAs were nucleofected into CD4+ T cells using an AMAXA Nucleofector apparatus (program U-14 or V-024; 3 μg of siRNA per 1 × 107 cells).

Infectivity Assays. LuSIV cells (2×10^4 cells/well) were seeded into 96-well. Cells were then disrupted with passive lysis buffer (Promega), and luciferase activity was measured using a reporter assay kit (Promega) 36 h after transfection. Transfections were performed in triplicate, and results were normalized to the Renilla luciferase signal. Each experiment was performed at least three times.

Western blots. Cells were washed with ice-cold PBS and then lysed on ice for 30 minutes with buffer containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5), and protease inhibitors (Roche). Protein lysates were clarified by centrifugation at 15,200 × g at 4°C for 20 minutes. Protein lysates were fractionated by SDS-PAGE, transferred to nitrocellulose, and detected by immunoblotting with an primary MAb at a 1:1,000 dilution. Immunoreactive proteins were detected using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG and an ECL assay (Amersham).

Virus purification and infection. Culture supernatants from chronically infected cell lines were collected 48 hours after transfection, centrifuged at 1,000 × g to remove cell debris, filtered through a 0.45-μm-pore-size filter, and concentrated by ultracentrifugation at 100,000 × g through a cushion of 20% sucrose in PBS. The pelleted virus was resuspended in RPMI with 10% FBS, aliquoted, and stored at −80°C. The viral titer was measured by anti-p24gag ELISA. Forty-eight hours after nucleofection with siRNA, CD4+ T cells were infected with virus (200 ng of p24 per 5×10^5 cells) by spinoculation

(1,200 × g, 2 h), followed by a 2-h incubation at 37°C, and were washed three times to remove input virus.

Differential lysis of chronically-infected cell lines and immunoprecipitation. Cells ($2x10^7$ cells/mL) were lysed in detergent (Brij97 with metal ions or triton-x) for 40 minutes on ice. Lysates were spun at 15,000 RPM for 20 minutes at 4ºC and the supernatant was reserved. Total protein in the supernatant was quantified by BCA assay. Lysate was pre-cleared with 10 uL protein A/G beads per 300ug protein, rotating 1 hr at 4ºC and then spun at 3,000 x g for 3 minutes at 4ºC. Supernatant was reserved. Lysate was adjusted to 1 mg/mL in appropriate buffer with protease inhibitors. Per sample, 1.5 ug primary antibody was incubated with 300 uL lysate on ice for 2 hours. Protein A/G beads (10 uL/sample) were added and the samples were rotated for 1 hr at 4ºC. Samples were spun at 3,000 x g for 3 minutes at 4ºC and the beads were reserved. Beads were washed three times in appropriate buffer. Remaining supernatant was removed using a 26½ guage needle. Beads were frozen for subsequent western blot analysis.

For immunoprecipitation, 50 µL 1X loading dye was added to each sample and samples were boiled (100ºC) for 5 minutes. Samples were quickly spun at 15,000 RPM and 20 µL sample was loaded on 10% Bis/Tris NuPage gels. For control, 20 µL cell lysates and 15 µL MN lysate (RIPA buffer) were used. For each sample 10 ug-70ug total protein was loaded per lane. Standard western blot procedures were followed, and blots were probed with human anti-HIV royal serum.

DR63 cell line generation. Jurkat cells (2×10^7) were suspended in 50 mL of medium containing 200 μg/mL ethyl methanesulfonate (Sigma) for 24 hours. Cells were washed 4 times in a medium containing 5% FBS and then cultured in complete medium for 7 days. Cells expressing reduced surface CD63levels were selected for through serial negative selection with anti-CD63 beads. Following six passages, cells were subcloned with limiting dilutions in 96-well plates (1-3 cells/well). When cell densities were at 1×10^5 or higher, cells were assessed for surface and intracellular CD63 levels using a plate-based adhesion assay (colorometric readout) and flow cytometry.

Results

As other groups have reported that the HIV lifecycle can be impacted by tetraspanin proteins, we attempted to determine whether these results were reproducible. We used siRNA medicated knockdown of tetraspanin or the tetraspanin-associted protein syntenin-1 to investigate viral release. As shown in Figure 4-2, we could achieve up to approximately 75% reduction in tetraspanin proteins by siRNA knockdown in chronically HIV_{JRCSF}-infected Jurkat cells. Additionally, we could simultaneously knockdown CD63 and CD82 expression. Knockdown of individual tetraspanins did not affect gag levels as determined by intracellular flow cytometry; however CD63/CD82 knockdown did decrease intracellular gag expression. As shown in Figures 4-3 and 4-4, siRNA knockdown of neither tetraspanins nor syntenin-1 impacted viral release.

We then assessed the phenotype of virions produced in these cells, hypothesizing that loss of reduction of tetrapsnin proteins had the potential to impact host protein incorporation into the viral envelope. Therefore, we assessed common integrin members of TEMs. While we could reduce or eliminate tetraspanin protein incorporation into the viral envelope, there were no observed changes in the incorporation of beta-1, beta-2 or alpha-5 integrins (Figure 4-5 and Figure 4-6).

As the incorporation of only three integrins were used to determine whether host protein incorporation had been altered due to tetraspanin siRNA, we assessed the overall function of the virions produced in these siRNA treated cells. Infectivity of virions produced in

Figure 4-2. Phenotype of Jurkat/HIV$_{JRCSF}$ cells following tetraspanins iRNA knockdown.

Jurkat/HIV$_{JRCSF}$ were nucleofected with siRNA against CD62, CD82, syntenin-1, an irrelevant control protein, or CD63 and CD82. Cellular expression of tetraspanin proteins (CD9, CD81, CD63, or CD82) and viral proteins (gag, p24) were assessed by flow cytometry following permeabilization. IgG was used as an isotype control.

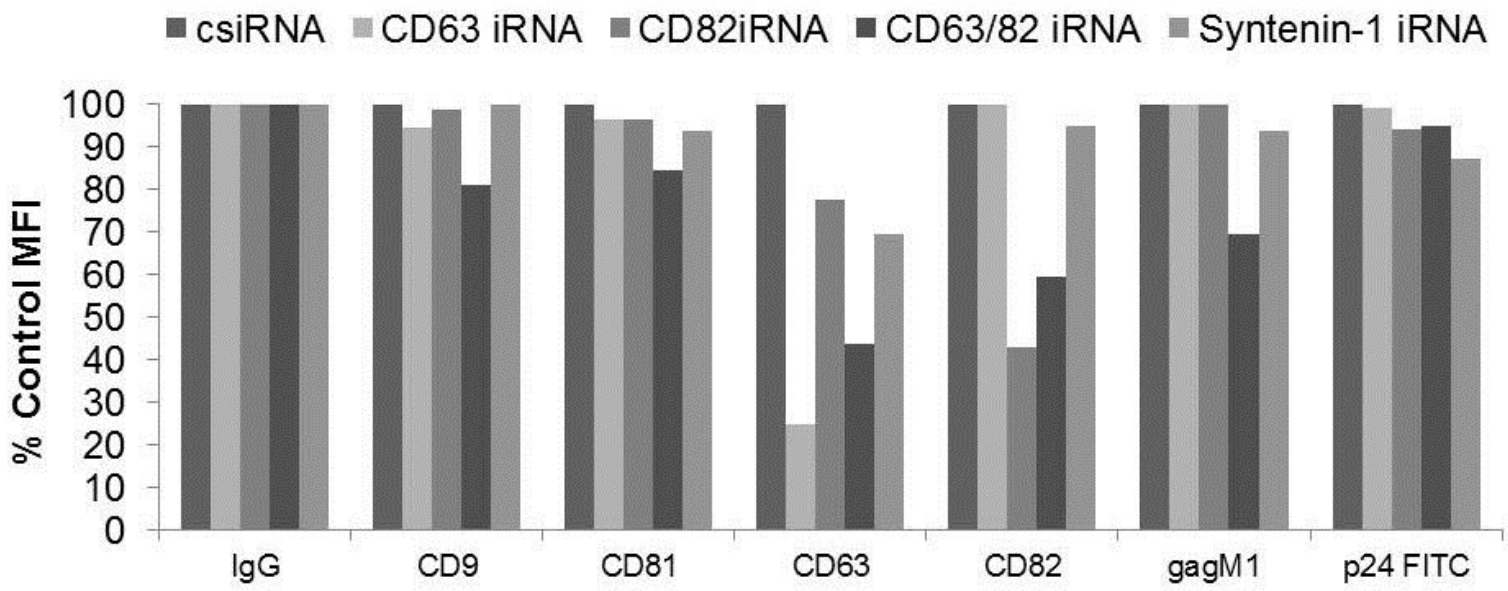

csiRNA
CD63 iRNA
CD82iRNA
CD63/82 iRNA
Syntenin-1 iRNA
% Control MFI
100
90
80
70
60
50
40
30
20
10
0
IgG
CD9
CD81
CD63
CD82
gagM1
p24 FITC

Figure 4-3. Tetraspanin iRNA effect on p24 release in Jurkat/HIV$_{JRCS}$F cells.

Supernatant was collected from Jurkat/HIV$_{JRCSF}$ cells which had been nucleofected with siRNAs against CD63, CD82, CD63/CD82, syntenin-1, or an irrelevant control protein. Total p24 was assessed in the supernatant and then adjusted for input cell number. Cell viability was also assessed.

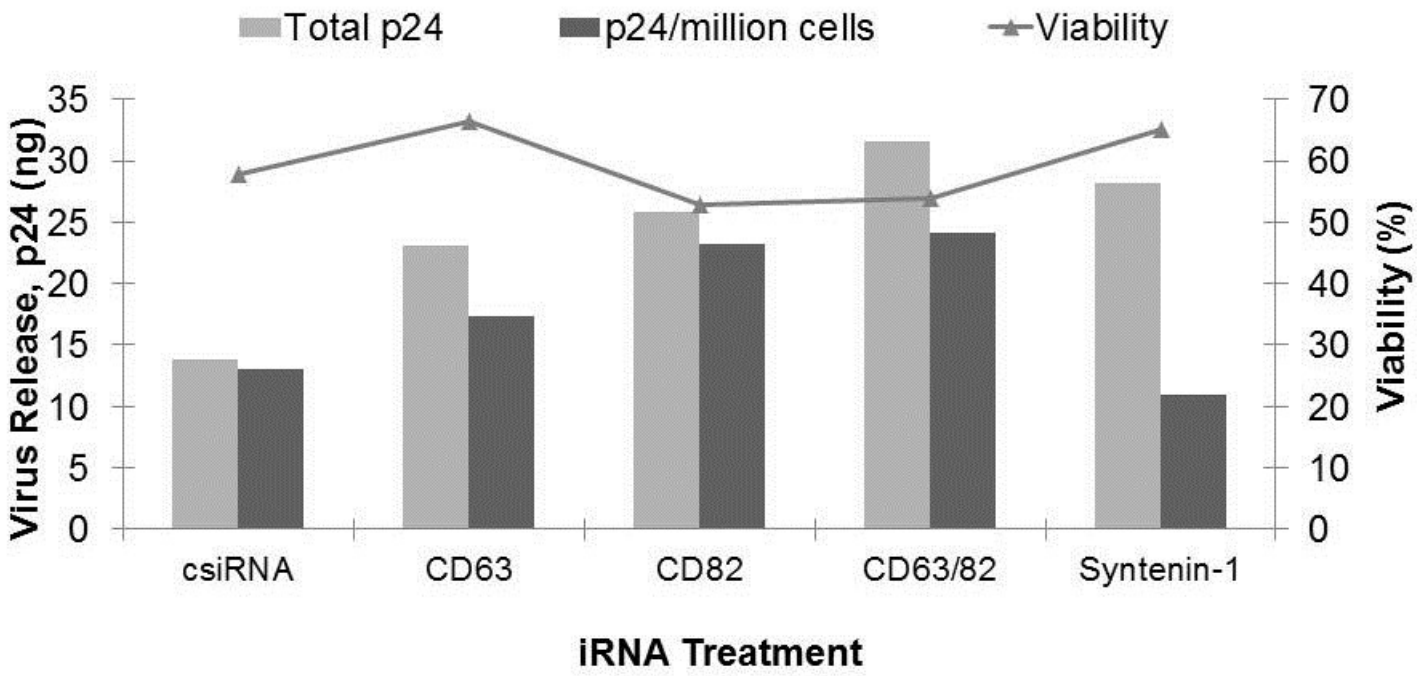

Total p24
p24/million cells
Viability
Virus Release, p24 (ng)
Viability (%)
0
5
10
15
20
25
30
35
0
10
20
30
40
50
60
70
csiRNA
CD63
CD82
CD63/82
Syntenin-1
iRNA Treatment

Figure 4-4. Tetraspanin iRNA effect on p24 release in PM1/HIV$_{IIIB}$ cells.

Supernatant was collected from PM1/HIV$_{IIIB}$ cells which had been nucleofected with siRNAs against CD63, CD82, CD63/CD82, or an irrelevant control protein. Total p24 was assessed in the supernatant and then adjusted for input cell number..

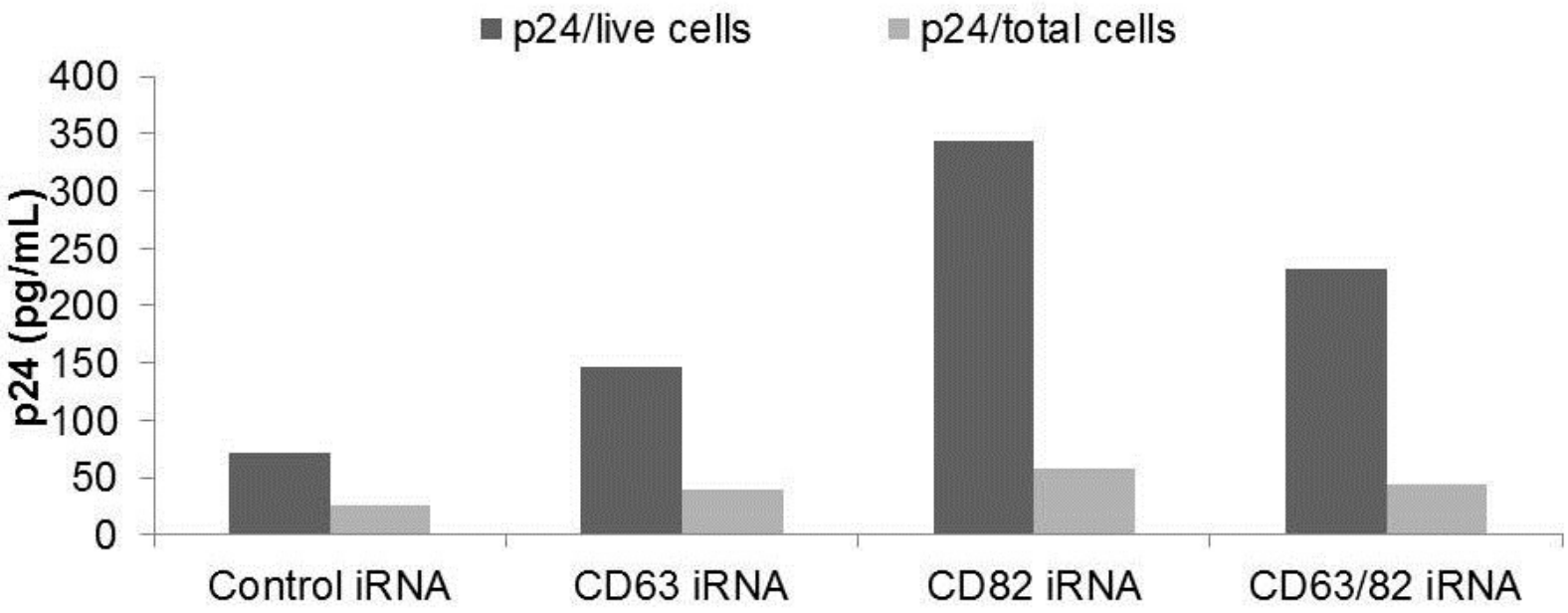

p24/live cells
p24/total cells
p24 (pg/mL)
400
350
300
250
200
150
100
50
0
Control iRNA
CD63 iRNA
CD82 iRNA
CD63/82 iRNA

Figure 4-5. Effect of tetraspanin iRNA knockdown in Jurkat/HIV$_{JRCSF}$ on HIV phenotype.

Purified viral particles collected from Jurkat/HIV$_{JRCSF}$ cells nucleofected with siRNA against CD63, CD82, CD63/CD82, or an irrelevant protein were immunoprecipitated with antibodies against common TEM-associated proteins. Viral phenotype was assessed by recovered p24 compared with input levels.

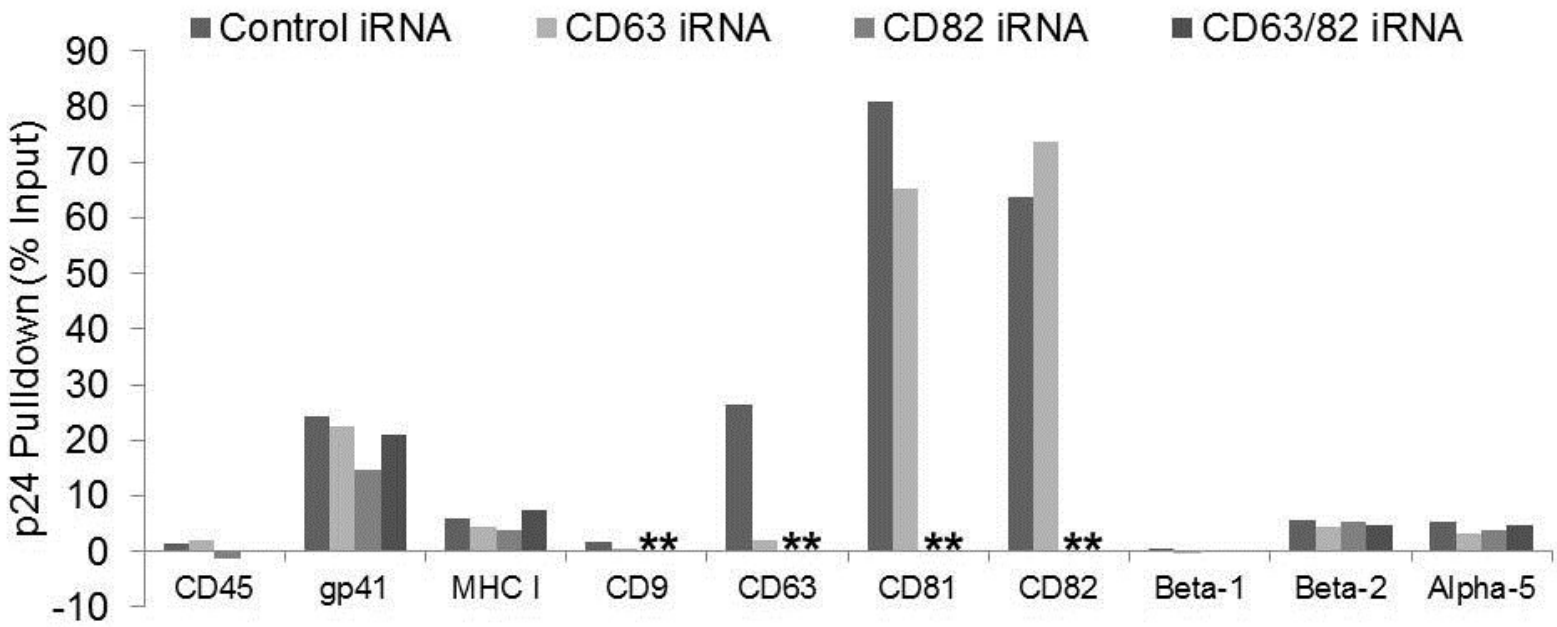
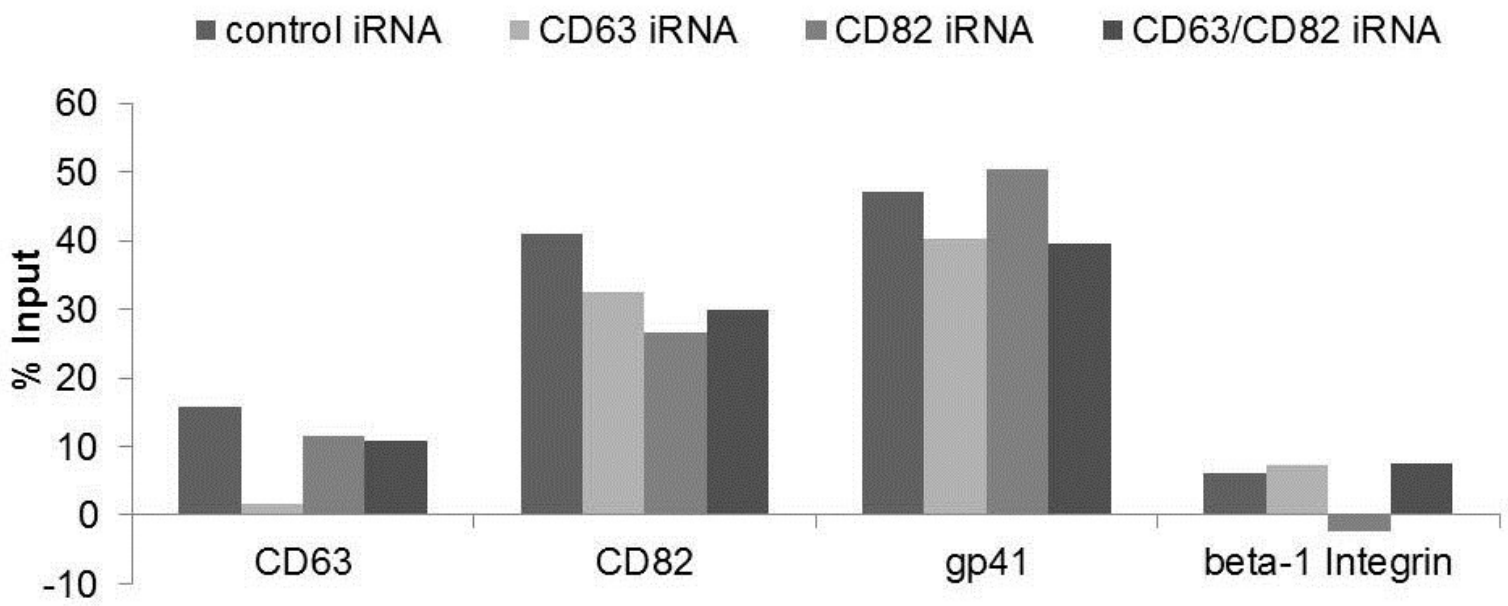

Control iRNA
CD63 iRNA
CD82 iRNA
CD63/82 iRNA
p24 Pulldown (% Input)
90
80
70
60
50
40
30
20
10
0
-10
**
**
**
**
CD45
gp41
MHC I
CD9
CD63
CD81
CD82
Beta-1
Beta-2
Alpha-5

Figure 4-6. Effect of tetraspanin iRNA knockdown in PM1/HIV$_{IIIB}$ on HIV phenotype.

Purified viral particles collected from PM1/HIV$_{IIIB}$ cells nucleofected with siRNA against CD63, CD82, CD63/CD82, or an irrelevant protein were immunoprecipitated with antibodies against common TEM-associated proteins. Viral phenotype was assessed by recovered p24 compared with input levels.


control iRNA
CD63 iRNA
CD82 iRNA
CD63/CD82 iRNA
% Input
60
50
40
30
20
10
0
-10
CD63
CD82
gp41
beta-1 Integrin

these iRNA treated cells was assessed. These viral stocks showed no difference to control viral stocks in terms of infectivity (Figure 4-7).

We also assessed the impact of blocking tetraspanins either on target cells or on virions, using monoclonal antibodies alone or in combination to pre-treat the target cells or virions. We found no effect on infectivity when pre-treating cells with mAbs to HIV (data not shown). We also saw no effect on infectivity when pre-treating virions with anti-tetraspanin mAbs (data not shown).

The lack of change in TEM associated proteins in the viral envelop and in the infectivity of these virions was unexpected, but we hypothesized that either functional overlap between the tetraspanins or the transient nature of siRNA knockdown could have impacted these results. Therefore, we created a cell line that had significantly reduced CD63 levels, with the intent of using this cell line in conjunction with siRNA or shRNA to knock down multiple tetraspanins and assess the impact on the viral lifecycle.

To create the CD63 downregulated cell line, cells were mutagenized with ethyl methanesulfonate (see Materials and Methods). Following mutagenesis, cells were serially passaged with negative selection for adhesion to anti-CD63 coated plates (Figure 4-8). After six passages, 75% of cells did not stably adhere to the plates. These cells were subcloned in limiting dilutions, with 1-3 cells per well.

Figure 4-7. Infectivity of HIV_{JRCSF} produced in iRNA treated cells.

The infectivity of viral particles produced from Jurkat cells nucleofected with siRNA against CD63, CD82, CD63/CD82, or an irrelevant protein was assessed through Lu-SIV assay, as described in the methods.

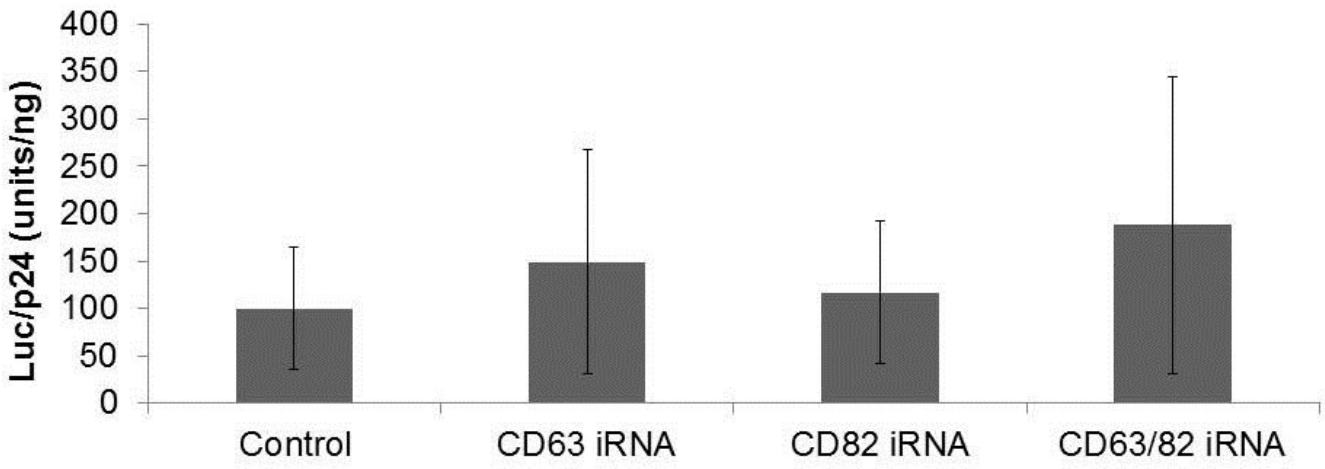
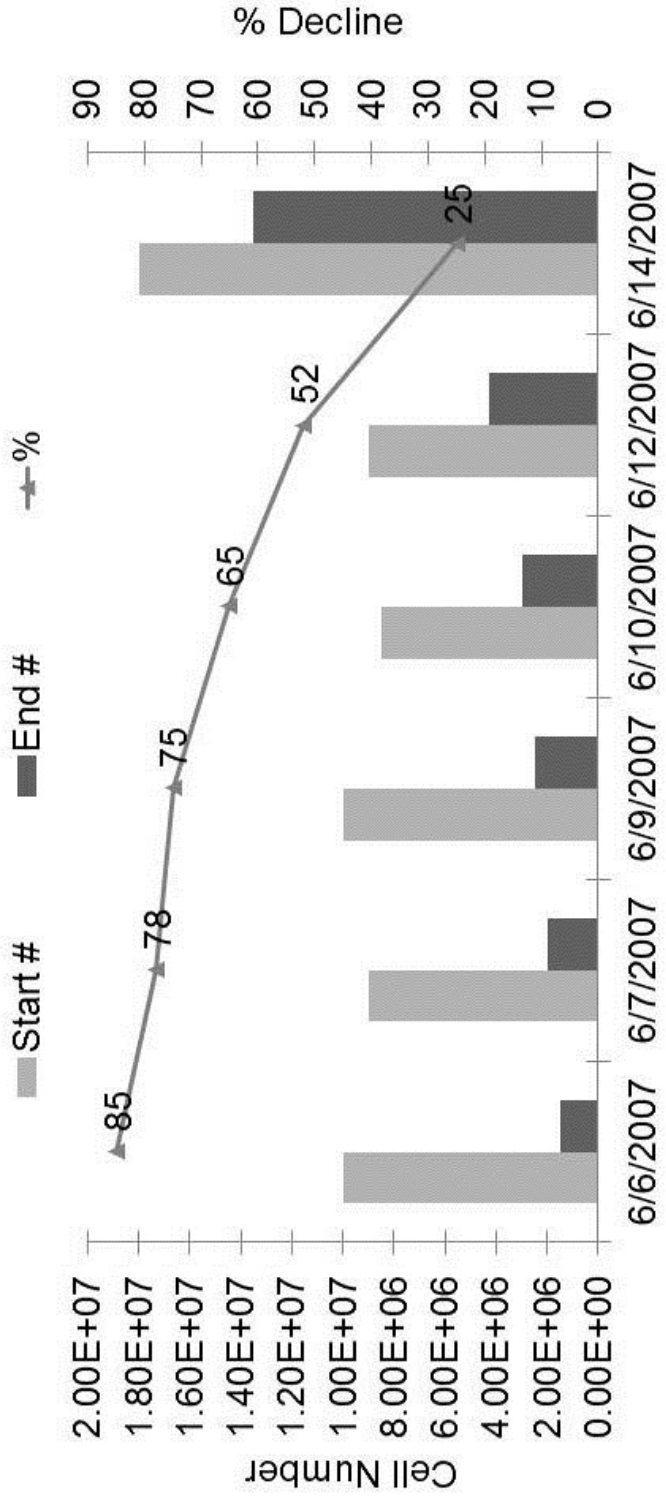

Luc/p24 (units/ng)
400
350
300
250
200
150
100
50
0
Control
CD63 iRNA
CD82 iRNA
CD63/82 iRNA

Figure 4-8. Selection against CD63 expression in mutagenized Jurkat cells.

Expression of CD63 in Jurkat cells mutagenized with EMS was selected against through sequential CD63 depletion in 96-well plates coated with anti-CD63 antibody. Enrichment of cells with putative depletion of cell surface CD63 was determined by the percentage of input cell number through the assay. Following the last depletion step, cells were subcloned and assessed for CD63 expression.


% Decline
90
80
70
60
50
40
30
20
10
0
Start #
End #
%
85
78
75
65
52
25
6/6/2007
6/7/2007
6/9/2007
6/10/2007
6/12/2007
6/14/2007
2.00E+07
1.80E+07
1.60E+07
1.40E+07
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
Cell Number

Subcloned cells were assayed for adhesion to anti-CD63 coated plates (Figure 4-9). Several clones that showed reduced adhesion compared to Jurkat controls were selected for further analysis. These clones were expanded and tested for CD63 expression by flow cytometry (Figure 4-10). Clone I5 was selected based on decrease extracellular and intracellular CD63 expression and reduced adhesion. This clone was renamed DR63 (for downregulated CD63).

As shown by fluorescent microscopy, DR63 cells showed reduced intracellular and intracellular CD63. While control Jurkat cells show CD63 in punctate clusters intracellularly, consistent with CD63 expression in intracellular vesicles, DR63 cells showed little to no CD63 in punctate clusters (Figure 4-11). Light microscopy showed that DR63s clustered and fused (Figure 4-12). Of interest, cells in which the CD63 partner syntenin-1 has been downregulated show a similar pattern of clustering and fusion.[223]

DR63 cells were infected with HIV_{JRCSF}, as described in the methods. Following infection, cells were washed and assessed for virus release. We observed no difference in viral release between control Jurkat cells and DR63 cells (Figure 4-13).

Figure 4-9. Subcloning of mutagenized Jurkat cells enriched for low cell surface CD63.

Mutagenized Jurkat cells that had been enriched for low CD63 cell surface levels were subcloned by limiting dilution. Subclones were assessed for cell surface CD63 by adhesion assay, using a standard colorometric readout (HRP/TMB) compared to unmutagenized Jurkat controls. Subclones were selected based on decreased intensity compared to control. Clones C3, C4, D3, F3, F4, F5, G4, G5, I5, K4, L2 and L3 were selected for further analysis of CD63 levels.

% Control
450
400
350
300
250
200
150
100
50
0
A1
A2
A3
A4
A5
A6
B1
B2
B3
B4
B5
B6
C1
C2
C3
C4
C5
C6
D1
D2
D3
D4
D5
D6
E1
E2
E3
E4
E5
E6
F1
F2
F3
F4
F5
F6
G1
G2
G3
G4
G5
G6
H1
H2
H3
H4
H5
H6
I1
I2
I3
I4
I5
I6
J1
J2
J3
J4
J5
J6
K1
K2
K3
K4
K5
K6
L1
L2
L3
L4
L5
L6
1/2 Input
Input
Jurkat 1
Jurkat 2

Figure 4-10. Subclone analysis of surface and intracellular CD63 expression levels. Subclones of interest were phenotyped for CD63 using flow cytometry. Surface CD63 levels were assessed in non-permeablized cells, while intracellular CD63 levels were assessed in permeablized cells. Flow cytometry results were compared with results from prior adhesion assays for the subclones. Clone I5 was selected for further analysis based on low cell surface and intracellular CD63 levels compared to unmutagenized Jurkat controls. This subclone was renamed DR63 (downregulated CD63).

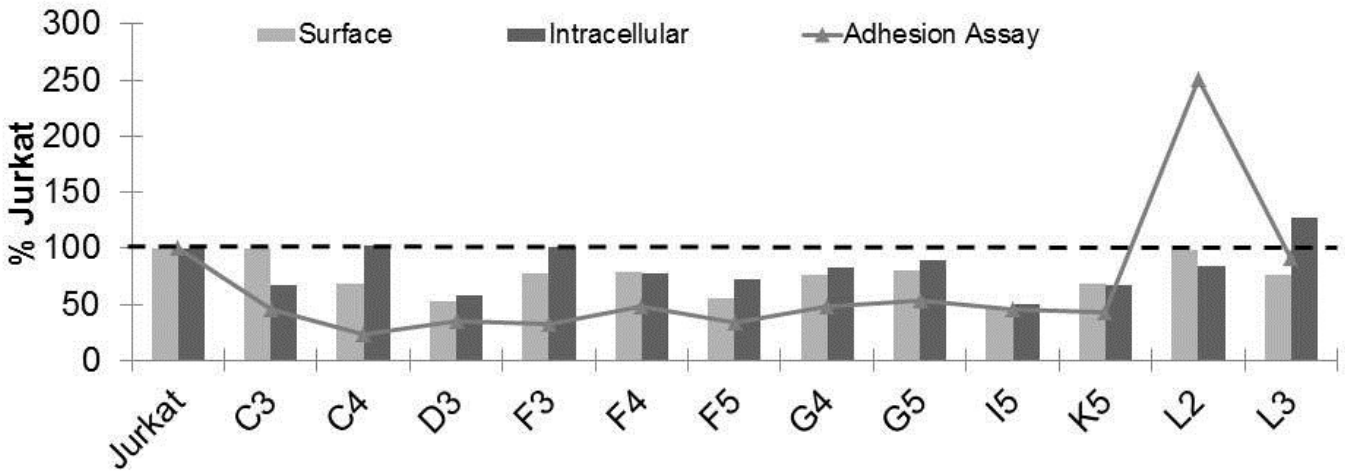

300
250
200
150
100
50
0
% Jurkat
Surface
Intracellular
Adhesion Assay
Jurkat
C3
C4
D3
F3
F4
F5
G4
G5
I5
K5
L2
L3

Figure 4-11. Fluorescent microscopy of DR63 cells.

Fluorescent microscopy assessed CD63-FITC stained (A) permeabilized unmutagenized Jurkat cells and (B) permeabilized DR63 cells. Normal Jurkat cells show both surface CD63, as well as CD63 in intracellular vesicles (presumably late endosomes). Comparatively, DR63 cells show significantly lower levels of surface CD63, as well as very low levels of intracellular CD63.

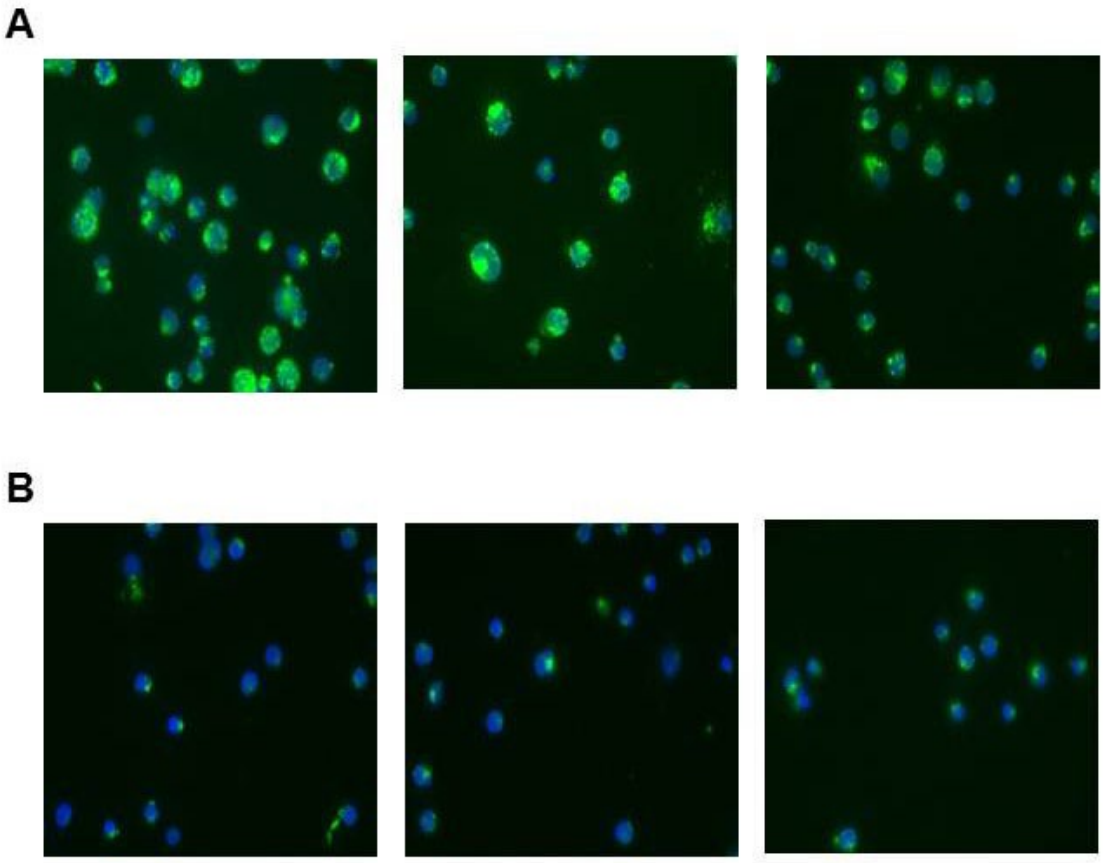

A
B

Figure 4-12. Light microscopy of DR63 cells.

DR63 cells show abnormal clumping and syncytia formation.

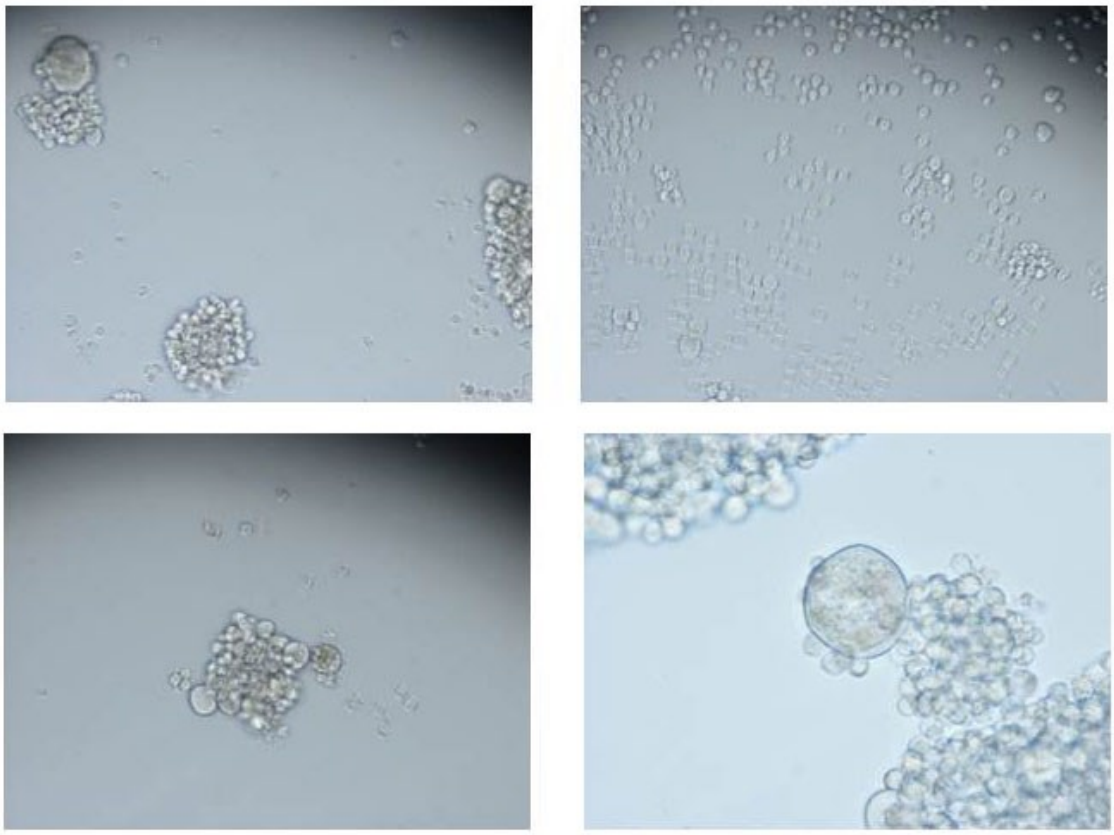
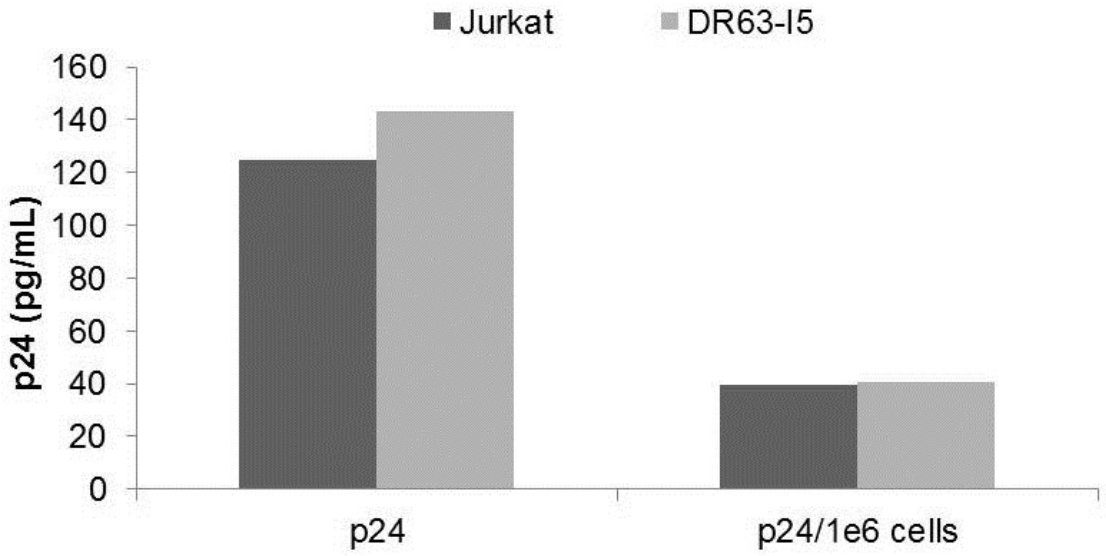


Figure 4-13. HIV_{JRCSF} release from HIV-infected Jurkat and DR63 cells.

Jurkat and DR63 cells were infected with HIV_{JRCSF} by spinoculation, as descried in the methods. 72 hours after infection, supernatant was collected and assessed or viral p24 levels.


Jurkat
DR63-I5
160
140
120
100
80
60
40
20
0
p24 (pg/mL)
p24
p24/1e6 cells

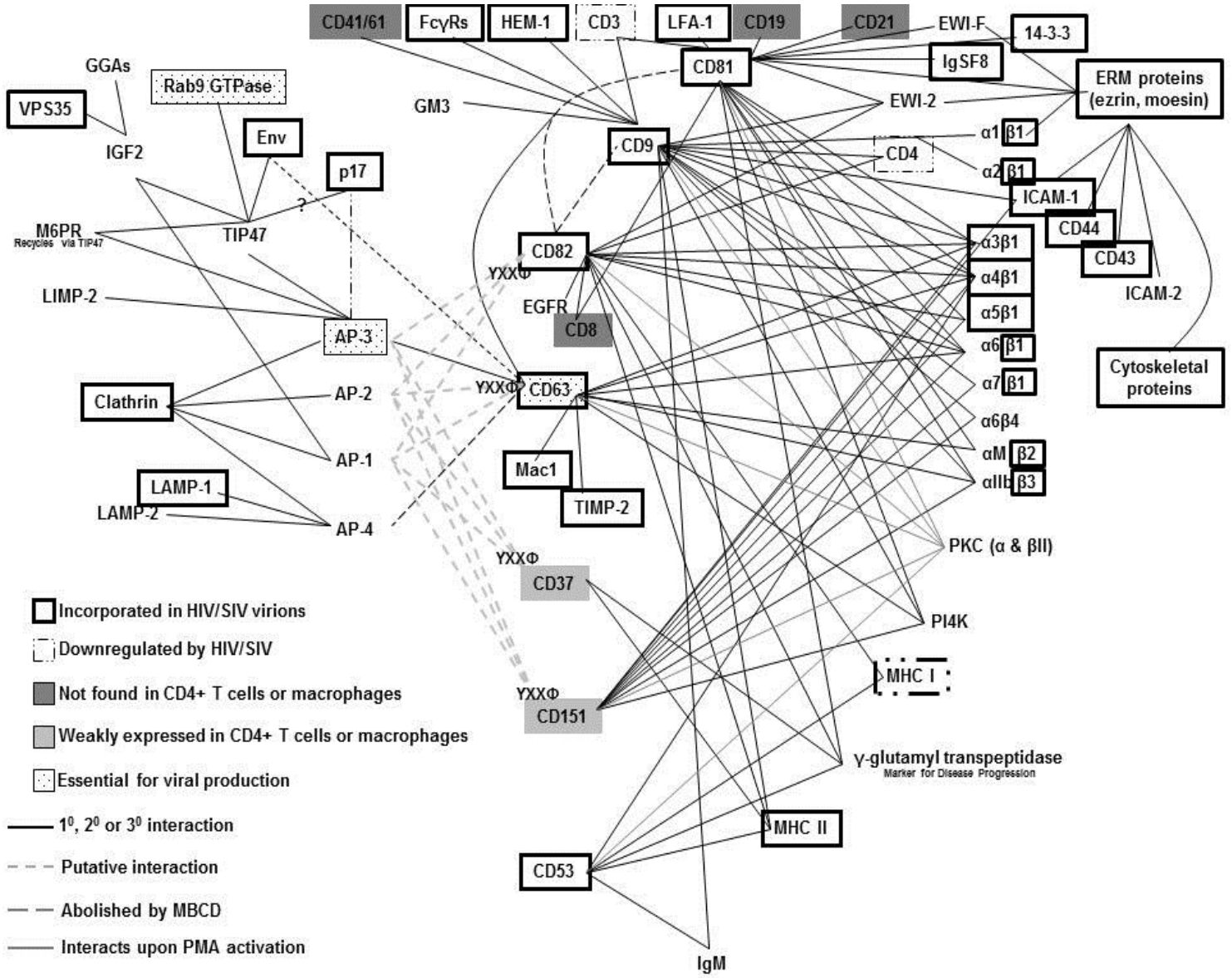
Discussion

Here, we found that disruption of tetraspanin expression by multiple methods in multiple cell types did not significantly affect virion infectivity, production, or incorporation of TEM resident proteins. This was unexpected, considering the reported importance of TEMs in HIV biology. However, the tetraspanin class of proteins has a considerable amount of structural and functional overlap and it is conceivable that we simply failed to target a sufficient number of TEM components to disrupt the viral lifecycle.

We had hypothesized that interactions between tetraspanins and adaptor proteins could drive host protein incorporation in the viral envelope (Figure 4-14). HIV env and gag have been shown to interact with the AP-2 complex during HIV assembly.[224] Adaptor proteins have also been shown to interact with tyrosine-based (Y-X-X-φ) motif.[225] Multiple tetraspanins include this motif on their c-terminal intracellular tails, suggesting that the tetraspanin proteins interact with adaptor proteins. CD63 has been shown to interact with the μ4, μ2, μ3a and μ3b subunits of AP-3; this interaction targets CD63 to the late endosomes/lysosomes.[226] Notably, this system bypasses the early endosomes.[226] AP-3 has also been found to interact with the HIV-1 matrix protein in a yeast two-hybrid system, but this interaction was not confirmed using nuclear magnetic resonance.[227,228] Hypothetically, this adaptor protein-tetraspanin interaction could serve as the bridge between viral proteins and the tetraspanin web. Indeed, it has been postulated by others that that the adaptor protein-viral protein interactions serve to localize viral budding from distinct microdomains.[224]

Figure 4-14. A hypothetical mechanism for the incorporation of TEM-associated proteins into HIV-1 particles.

In this hypothesis, TEM-resident proteins are incorporated into the viral envelope via interaction with tetraspanin proteins. Tetraspanin proteins interact with adapter protein complexes via a tyrosine-based motif in the C-terminus. Adapter protein complexes are proposed to interact directly with viral proteins, as well as indirect interactions. Protein interactions are drawn from the literature.[119,159,161,198,229-231]


CD41/61
FcγRs
HEM-1
CD3
LFA-1
CD19
CD21
EWI-F
14-3-3
CD81
IgSF8
ERM proteins (ezrin, moesin)
GGAs
Rab9 GTPase
VPS35
GM3
EWI-2
IGF2
Env
CD9
α1 β1
CD4
α2 β1
p17
ICAM-1
CD44
M6PR
Recycles via TIP47
TIP47
CD82
α3β1
CD43
YXXΦ
α4β1
LIMP-2
EGFR
α5β1
ICAM-2
CD8
AP-3
α6 β1
Cytoskeletal proteins
YXXΦ
CD63
α7 β1
Clathrin
AP-2
α6β4
αM β2
AP-1
Mac1
αIIb β3
LAMP-1
TIMP-2
LAMP-2
AP-4
PKC (α & βII)
YXXΦ
CD37
PI4K
MHC I
YXXΦ
CD151
γ-glutamyl transpeptidase
Marker for Disease Progression
MHC II
CD53
IgM
Incorporated in HIV/SIV virions
Downregulated by HIV/SIV
Not found in CD4+ T cells or macrophages
Weakly expressed in CD4+ T cells or macrophages
Essential for viral production
1⁰, 2⁰ or 3⁰ interaction
Putative interaction
Abolished by MBCD
Interacts upon PMA activation

There are several ways in which HIV may be targeted to TEMs. A number of HIV proteins have been reported to directly interact with cytoskeletal proteins and ERM proteins have been reported to be incorporated into the virus as well. The CD9 and CD81 interacting partners EWI-2 and EWI-F have been reported to interact with ERM proteins.[230] For HTLV-1, Mazurov and colleagues reported that the matrix domain of the gag protein concentrates at TEMs, interacting with CD82 and CD81.[178,179] This interaction is dependent on the conserved amino acids on the short CD82 intracellular loop or the conserved cysteines flanking the loop.[178] They also determined that the matrix domain interacts with CD81 inner loop. To determine if there is an interaction between tetraspanins and HIV, tetraspanins were immunoprecipitated from infected cells. The immunoiprecipitant was then probed for all HIV proteins using royal human anti-HIV antibody via western blot, as described in the Materials and Methods. These blots were striped and re-probed using a monoclonal antibody against HIV p17 (matrix). For HIV, we found no evidence of direct interaction between p17 and CD9, CD83, CD81 or CD82 (data not shown).

The C-terminal tail of CD63 has also been shown to interact with syntenin-1, through the syntenin-1 PDZ domain.[232] We have shown that syntenin-1 is incorporated in viral particles produced in both T cell and MDM cells. When treating chronically infected producer cells with a syntenin-1 siRNA, we found a small decrease in viral release compared to control and a decrease in cellular CD63. Syntenin-1 may represent a potential TEM access point for HIV, although no direct interaction between the molecule and viral proteins have been discovered. However, syntenin-1 has also been shown to

interact with ALIX and be of importance in exosome formation, so it may be possible that its inclusion in the viral particle merely represents HIV entry into the exosome biogenesis pathway.[233] It is interesting to note that syntenin-1 plays a role in cell-cell adhesion in epithelial cells and knockdown of syntenin-1 has been shown to result in cell aggregation.[223] In the DR63 cell line, we also observed cell clumping, as well as cell fusion. This has potential implications for HIV particles, which are required to fuse with plasma membranes during the viral lifecycle.

One group interested in customizing retroviral-like particles for the purpose of drug delivery produced viral like particles (VLPs) in cells containing shRNA against CD81. The cells, which had 99% reduction in CD81 levels, produced VLPs that were CD81 deficient. The group did not investigate the effects of CD81 knockdown on the incorporation of other host proteins in the VLPs; however, it appears that knocking down CD81 in this cell line (293 derivatives) did not negatively affect VLP release.[234] Similarly, we did not detect any significant differences in viral release, infectivity, or phenotype in these studies when knocking down CD63 or CD82. However, given the multiple studies from multiple groups reporting the importance of tetraspanins and TEMs in the HIV lifecycle, we believe that the lack of effect shown here may be due in part to functional redundancy within the tetraspanin class. Further, if this class of proteins and their associated cellular partners are of vital importance in the HIV lifecycle, HIV may have developed multiple access points targeting TEMs, as described in "Chapter V: Hypothetical Determinants of the HIV Envelope Composition".

CHAPTER V

HYPOTHETICAL DETERMINANTS OF THE HIV ENVELOPE COMPOSITION

There are a few basic requirements that drive viral evolution and, consequently, viral phenotype. First, the virus must be able to escape host restriction factors. For HIV, the accessory proteins vif and vpu (and possibly others) serve this function. These proteins bind ABOBEC3G and tetherin, respectively, allowing for productive viral infection.[5,6] Thus, some primary interactions between viral proteins and host proteins have been selected for based on host restriction pressure. In our studies, we found that HIV produced in T cells and macrophages incorporate the protein α1-antitrypsin (AAT), which blocks the cleavage of gp160 and the gag polyprotein. It has been shown that the HIV protease binds and cleaves AAT.[140-142] Thus, AAT incorporation into the viral particle likely results from selective host pressure and AAT may represent a host restriction factor.

For enveloped viruses, there is also the requirement that viral particles be capable of membrane fission and fusion. Microvesicles (e.g., exosomes) and HIV particles appear to have similar host protein and lipid compositions.[58,152,235,236] These small particles tend to be enriched for in sphingolipids, cholesterol, tetraspanins, syntenin-1, and other proteins described in detail above. Further, these particles utilize similar host processes for generation.[233,237] These common factors may reflect a need for both certain structural features, such as a membrane that can accommodate a greater curvature than is found in the cell membrane, as well as functional ability, including the potential to fuse with other membranes. Indeed, there cannot be unlimited methods of fission and fusion of lipid bilayers and there are likely specific biophysical requirements to accommodate the thermodynamic requirements necessary for these processes. Therefore, it should not be

surprising that HIV co-opts the normal cellular fission and fusion processes and the lipids and proteins involved are thus of vital importance in the viral lifecycle.

It follows that the host protein composition of the viral particle would reflect these processes and that the viral envelope would mirror that of microvesicles or exosomes. As it is well established that HIV buds from lipid ordered domains, particularly lipid rafts and TEMs, we find many of the host proteins characterized in these cellular domains in the viral envelope. It is important to recognize that the virus "taps in" to these domains, and that this is likely the result of a few primary viral interactions. The literature has shown that HIV can interact with literally hundreds of proteins; in reality, many of these are most likely not primary interactions. The HIV genome only encodes for 16 proteins and these proteins can only make a finite number of primary interactions. Biochemical analyses are prone to produce artifacts indicating primary interactions when no interaction may occur. For example, a yeast two-hybrid system was used to show a primary interaction between AP-3 and gag;[227] further analysis by NMR could not reproduce this result.[228] What is notable about this example is that the original paper, published in the highly respected journal *Cell*, seemed to have strong evidence for the interaction, but the *same group* could not find this interaction using alternate methods. Similarly, many of the interactions reported to occur (or not to occur) may be a result of assay artifact or experimental conditions. Identification of true primary interactions is particularly important as a result, as these interactions not only arise due to selective pressure, but they are likely to have significant importance in the HIV lifecycle.

In an effort to characterize these interactions that may be of importance in the HIV-1 lifecycle, we identified host proteins in virions produced in both CD4+ T cell lines and MDMs. We hypothesized that incorporated host proteins of importance would not be dependent of producer cell type, although there certainly could be instances where important host proteins are cell type dependent. In our model, the common set of proteins incorporated into HIV-1 particles regardless of producer cell type serve as protein hubs, determining the viral phenotype through their primary and higher order interactions with other cellular proteins. This would not only drive differences in virion phenotype between cell types (as the host-host protein interactions will vary by cell type), but it would also likely drive differences in virion phenotype produced in the same cell types, and even from the same producer cell. Spatial and temporal differences in protein interactions would produce a heterogeneous viral phenotype regardless of budding circumstances. Consequently, this may account for the large number of incorporated proteins reported to be incorporated into HIV-1 particles.

It remains speculative, but the inclusion of specific host proteins may result from the need to include lipids that are capable of fission and fusion. In the case of membrane fission (budding), it has been shown that membrane-associated proteins can induce bilayer deformation, which may result in protein aggregation.[238] Specific lipids or cholesterol in small clusters can themselves behave effectively as membrane inclusions.[238] By selecting proteins that have a favorable geometric mismatch—that is, membrane proteins that cause a curvature in lipid bilayer—HIV particles may be able to induce membrane deformations that are essential for the budding process. Thus, HIV may

use established host protein-protein networks (i.e., TEMs) to control membrane deformations. The identified protein syntinin-1 interacts with the protein ALIX and is a core component of exosome biogenesis.[233] HIV-1 incorporation of syntenin-1 in both T cells and MDMs supports the suggestion that HIV targets the exosome biogenesis machinery during viral egress, regardless of cell type. It is interesting that syntenin-1 appears to have a late-domain-like motif (the N-terminal domain of syntenin contains three LYPX(n)L motifs) similar to HIV p6 and EIAV p9.[237,239] Syntenin-1 is also a known component of TEMs and it interacts with the PDZ domain of CD63 and may be involved in CD63 trafficking between internal membranes and the plasma membrane.[232] Given the importance of syntenin-1 in exosome formation and interaction with the ESCRT machinery, as well as its primary interaction with CD63 (as shown by NMR), it is likely that the molecule is of importance in both HIV release, as well as in determining the viral phenotype.

HIV particles must also be able to fuse with target cells. In the hemifusion (stalk) model of lipid membrane fusion, the stalk must be metastable in order to allow for fast fusion, otherwise fusion is either extremely slow or impossible.[240] Accordingly, the variation allowed in the membrane lipid architecture that would allow for a metastable stalk is small. To achieve membrane fusion, lipid bilayers could be composed of lipids with small spontaneous curvatures, or lamellar formers, and those with larger negative spontaneous curvatures, or nonlamellar formers. The ratio of these different kinds of lipids must be tightly regulated, as is observed for *Escherichia coli* and *Acholeplasma laidlawii*.[240-242] HIV must also tightly control its lipid composition to retain a lipid

composition that remains capable of fusion with target cells. Our group has previously shown that cholesterol in the viral envelop is necessary for viral fusion.[54]

HIV must also target membrane regions of cells that contain regions amenable to membrane fusion. The HIV fusion protein gp41 cholesterol binding is attributed to a cholesterol recognition consensus (CRAC) motif, which mediates gp41 binding to a cholesterol affinity column.[243] Based on thermodynamics and steric properties, the transmembrane regions of certain proteins may more readily accommodate association with small, planar lipids. Notably, TEMs, which have four transmembrane regions, are enriched in lipids with these qualities. Additionally, HIV coreceptors are seven-pass transmembrane proteins located in lipid ordered domains, which appears to be a requirement for HIV fusion with target cells.[244-246] It has been proposed that localization of coreceptors in lipid ordered domains is needed for signaling events to establish productive infection of a cell.[247] However, it also seems reasonable that localization of these proteins in lipid ordered domains is necessitated by the thermodynamic requirements of membrane fusion. Of note, other enveloped viruses also target multipass transmembrane proteins during viral entry. Many other viral species also require cholesterol for fusion and have receptors located within lipid ordered domains (Table 5-1). Thus, the membrane fission and fusion requirements are linked—at egress, viral particles must have a lipid and protein composition that allows for later fusion. They must then target lipid ordered regions for fusion. These requirements would be similar for any viral species with an envelope and would, therefore, constitute a selective pressure on all enveloped viruses. Accordingly, it may not be surprising that some of the host proteins

Table 5-1. Receptor Characteristics for Selected Enveloped Viruses.

Virus	Receptor or Coreceptor	Found in Lipid-ordered Domains	Viral fusion requires cholesterol?
CMV[248,249]	α2β1; α6β1; α5β3	Y	Y
EBV[250,251]	CD21; CD35; HLA II	Y	Y
HBV[252]	Still undefined	--	--
HCV	CD81, LDL	Y	Y
HSV[253-256]	Heparan Sulfate; HVEM, Nectin-1; Nectin-2	Y	Y
HIV[55]	CD4; CCR5; CXCR4	Y	Y
VZV[257,258]	M6P	Y	Y

identified as HIV components (e.g., syntenin-1, HSP90, ezrin, tetraspanins, and annexin A2) are also found in mass spectrometry analyses from other virions (such as Newcastle disease virus, PRRSV, and influenza virus).[259-261] It must be noted, however, that these similarities are merely thought-provoking and it remains to be shown whether there are commonalities between enveloped viruses that drive them to target similar host processes during egress and entry.

As there is a signal that fission and fusion selective pressures appear to be shared across multiple viruses, we can speculate that greater insight could impact treatment for many viral infections. Of course, as these processes are also vital for proper cellular function, targeting these processes directly many not be clinically viable. Targeting viral-host protein interactions; however, may be a reasonable goal. Considering the implications of primary interactions between viral proteins and host proteins and the impact on viral budding and membrane fusion, identifying the interactions that drive the viral phenotype could provide important insights into methods of treatment.

References

1. World Health Organization. HIV/AIDS. 2013; http://www.who.int/gho/hiv/en/index.html. Accessed March 10, 2013.
2. Grewe B, Uberla K. The human immunodeficiency virus type 1 Rev protein: menage a trois during the early phase of the lentiviral replication cycle. *The Journal of general virology.* Aug 2010;91(Pt 8):1893-1897.
3. Vermeire J, Vanbillemont G, Witkowski W, Verhasselt B. The Nef-infectivity enigma: mechanisms of enhanced lentiviral infection. *Current HIV research.* Oct 2011;9(7):474-489.
4. Ott M, Geyer M, Zhou Q. The control of HIV transcription: keeping RNA polymerase II on track. *Cell host & microbe.* Nov 17 2011;10(5):426-435.
5. Neil SJ, Zang T, Bieniasz PD. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature.* Jan 24 2008;451(7177):425-430.
6. Bishop KN, Holmes RK, Sheehy AM, Malim MH. APOBEC-mediated editing of viral RNA. *Science.* Jul 30 2004;305(5684):645.
7. Jowett JB, Planelles V, Poon B, Shah NP, Chen ML, Chen IS. The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. *J Virol.* Oct 1995;69(10):6304-6313.
8. Ayyavoo V, Mahboubi A, Mahalingam S, et al. HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor kappa B. *Nature medicine.* Oct 1997;3(10):1117-1123.
9. Stewart SA, Poon B, Jowett JB, Chen IS. Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest. *J Virol.* Jul 1997;71(7):5579-5592.
10. Goh WC, Rogel ME, Kinsey CM, et al. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. *Nature medicine.* Jan 1998;4(1):65-71.
11. Muthumani K, Hwang DS, Desai BM, et al. HIV-1 Vpr induces apoptosis through caspase 9 in T cells and peripheral blood mononuclear cells. *J Biol Chem.* Oct 4 2002;277(40):37820-37831.
12. Muthumani K, Choo AY, Zong WX, et al. The HIV-1 Vpr and glucocorticoid receptor complex is a gain-of-function interaction that prevents the nuclear localization of PARP-1. *Nature cell biology.* Feb 2006;8(2):170-179.
13. U.S. Food and Drug Administration. Antiretroviral drugs used in the treatment of HIV infection. 2013; http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm. Accessed March 10, 2013.
14. Resh MD. The ubiquitous nature of budding. *Trends Microbiol.* 2001;9(2):57.
15. Freed EO. Viral late domains. *J Virol.* 2002;76(10):4679-4687.
16. Goff A, Ehrlich LS, Cohen SN, Carter CA. Tsg101 control of human immunodeficiency virus type 1 Gag trafficking and release. *J Virol.* 2003;77(17):9173-9182.
17. Garrus JE, von Schwedler UK, Pornillos OW, et al. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell.* 2001;107(1):55-65.

18. Ott DE, Coren LV, Sowder RC, 2nd, Adams J, Schubert U. Retroviruses have differing requirements for proteasome function in the budding process. *J Virol.* 2003;77(6):3384-3393.
19. Stahl PD, Barbieri MA. Multivesicular bodies and multivesicular endosomes: the "ins and outs" of endosomal traffic. *Sci STKE.* 2002;2002(141):PE32.
20. Bache KG, Brech A, Mehlum A, Stenmark H. Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. *J Cell Biol.* 2003;162(3):435-442.
21. Katzmann DJ, Stefan CJ, Babst M, Emr SD. Vps27 recruits ESCRT machinery to endosomes during MVB sorting. *J Cell Biol.* 2003;162(3):413-423.
22. Bishop N, Horman A, Woodman P. Mammalian class E vps proteins recognize ubiquitin and act in the removal of endosomal protein-ubiquitin conjugates. *J Cell Biol.* 2002;157(1):91-101.
23. Dupre S, Volland C, Haguenauer-Tsapis R. Membrane transport: ubiquitylation in endosomal sorting. *Curr Biol.* 2001;11(22):R932-934.
24. Bishop N, Woodman P. TSG101/mammalian VPS23 and mammalian VPS28 interact directly and are recruited to VPS4-induced endosomes. *J Biol Chem.* 2001;276(15):11735-11742.
25. Pike LJ. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res.* Jul 2006;47(7):1597-1598.
26. Nguyen DH, Hildreth JE. Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J Virol.* Apr 2000;74(7):3264-3272.
27. Lindwasser OW, Resh MD. Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane microdomains. *J Virol.* Sep 2001;75(17):7913-7924.
28. Ono A, Freed EO. Plasma membrane rafts play a critical role in HIV-1 assembly and release. *Proc Natl Acad Sci U S A.* Nov 20 2001;98(24):13925-13930.
29. Hermida-Matsumoto L, Resh MD. Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. *J Virol.* 2000;74(18):8670-8679.
30. Brugger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, Krausslich HG. The HIV lipidome: a raft with an unusual composition. *Proc Natl Acad Sci U S A.* Feb 21 2006;103(8):2641-2646.
31. Brown DA, London E. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol.* 1998;14:111-136.
32. Brown DA, London E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem.* Jun 9 2000;275(23):17221-17224.
33. London E, Brown DA. Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim Biophys Acta.* Nov 23 2000;1508(1-2):182-195.
34. Schroeder RJ, Ahmed SN, Zhu Y, London E, Brown DA. Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. *J Biol Chem.* Jan 9 1998;273(2):1150-1157.

35. Robbins SM, Quintrell NA, Bishop JM. Myristoylation and differential palmitoylation of the HCK protein-tyrosine kinases govern their attachment to membranes and association with caveolae. *Mol Cell Biol.* Jul 1995;15(7):3507-3515.

36. Solomon KR, Mallory MA, Finberg RW. Determination of the non-ionic detergent insolubility and phosphoprotein associations of glycosylphosphatidylinositol-anchored proteins expressed on T cells. *Biochem J.* Sep 1 1998;334 (Pt 2):325-333.

37. Gheysen D, Jacobs E, de Foresta F, et al. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell.* Oct 6 1989;59(1):103-112.

38. Jacobs E, Gheysen D, Thines D, Francotte M, de Wilde M. The HIV-1 Gag precursor Pr55gag synthesized in yeast is myristoylated and targeted to the plasma membrane. *Gene.* Jun 30 1989;79(1):71-81.

39. Ono A, Freed EO. Binding of human immunodeficiency virus type 1 Gag to membrane: role of the matrix amino terminus. *J Virol.* May 1999;73(5):4136-4144.

40. Ono A, Orenstein JM, Freed EO. Role of the Gag matrix domain in targeting human immunodeficiency virus type 1 assembly. *J Virol.* Mar 2000;74(6):2855-2866.

41. Dick RA, Goh SL, Feigenson GW, Vogt VM. HIV-1 Gag protein can sense the cholesterol and acyl chain environment in model membranes. *Proc Natl Acad Sci U S A.* Nov 13 2012;109(46):18761-18766.

42. Freed EO, Martin MA. Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. *J Virol.* Jan 1996;70(1):341-351.

43. Freed EO, Martin MA. Evidence for a functional interaction between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp120. *J Virol.* Apr 1994;68(4):2503-2512.

44. Freed EO, Martin MA. The role of human immunodeficiency virus type 1 envelope glycoproteins in virus infection. *J Biol Chem.* Oct 13 1995;270(41):23883-23886.

45. Freed EO, Martin MA. Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix. *J Virol.* Mar 1995;69(3):1984-1989.

46. Freed EO, Orenstein JM, Buckler-White AJ, Martin MA. Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. *J Virol.* Aug 1994;68(8):5311-5320.

47. Hourioux C, Brand D, Sizaret PY, et al. Identification of the glycoprotein 41(TM) cytoplasmic tail domains of human immunodeficiency virus type 1 that interact with Pr55Gag particles. *AIDS Res Hum Retroviruses.* Aug 10 2000;16(12):1141-1147.

48. Bhattacharya J, Repik A, Clapham PR. Gag regulates association of human immunodeficiency virus type 1 envelope with detergent-resistant membranes. *J Virol.* Jun 2006;80(11):5292-5300.

49. Brugger B, Krautkramer E, Tibroni N, et al. Human immunodeficiency virus type 1 Nef protein modulates the lipid composition of virions and host cell membrane microdomains. *Retrovirology.* 2007;4:70.
50. Guyader M, Kiyokawa E, Abrami L, Turelli P, Trono D. Role for human immunodeficiency virus type 1 membrane cholesterol in viral internalization. *J Virol.* 2002;76(20):10356-10364.
51. Campbell S, Gaus K, Bittman R, Jessup W, Crowe S, Mak J. The raft-promoting property of virion-associated cholesterol, but not the presence of virion-associated Brij 98 rafts, is a determinant of human immunodeficiency virus type 1 infectivity. *J Virol.* Oct 2004;78(19):10556-10565.
52. Viard M, Parolini I, Sargiacomo M, et al. Role of cholesterol in human immunodeficiency virus type 1 envelope protein-mediated fusion with host cells. *J Virol.* 2002;76(22):11584-11595.
53. Campbell SM, Crowe SM, Mak J. Virion-associated cholesterol is critical for the maintenance of HIV-1 structure and infectivity. *Aids.* 2002;16(17):2253-2261.
54. Liao Z, Graham DR, Hildreth JE. Lipid rafts and HIV pathogenesis: virion-associated cholesterol is required for fusion and infection of susceptible cells. *AIDS Res Hum Retroviruses.* Aug 2003;19(8):675-687.
55. Graham DR, Chertova E, Hilburn JM, Arthur LO, Hildreth JE. Cholesterol depletion of human immunodeficiency virus type 1 and simian immunodeficiency virus with beta-cyclodextrin inactivates and permeabilizes the virions: evidence for virion-associated lipid rafts. *J Virol.* Aug 2003;77(15):8237-8248.
56. Ono A, Waheed AA, Freed EO. Depletion of cellular cholesterol inhibits membrane binding and higher-order multimerization of human immunodeficiency virus type 1 Gag. *Virology.* Mar 30 2007;360(1):27-35.
57. Hogue IB, Grover JR, Soheilian F, Nagashima K, Ono A. Gag induces the coalescence of clustered lipid rafts and tetraspanin-enriched microdomains at HIV-1 assembly sites on the plasma membrane. *J Virol.* Oct 2011;85(19):9749-9766.
58. Gould SJ, Booth AM, Hildreth JE. The Trojan exosome hypothesis. *Proc Natl Acad Sci U S A.* Sep 16 2003;100(19):10592-10597.
59. Rodgers W, Rose JK. Exclusion of CD45 inhibits activity of p56lck associated with glycolipid-enriched membrane domains. *J Cell Biol.* 1996;135(6 Pt 1):1515-1523.
60. Nusrat A, Parkos CA, Verkade P, et al. Tight junctions are membrane microdomains. *J Cell Sci.* 2000;113(Pt 10):1771-1781.
61. Tremblay MJ, Fortin JF, Cantin R. The acquisition of host-encoded proteins by nascent HIV-1. *Immunology today.* Aug 1998;19(8):346-351.
62. Cantin R, Methot S, Tremblay MJ. Plunder and stowaways: incorporation of cellular proteins by enveloped viruses. *J Virol.* Jun 2005;79(11):6577-6587.
63. Luban J, Bossolt KL, Franke EK, Kalpana GV, Goff SP. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell.* Jun 18 1993;73(6):1067-1078.
64. Franke EK, Yuan HE, Luban J. Specific incorporation of cyclophilin A into HIV-1 virions. *Nature.* Nov 24 1994;372(6504):359-362.

65. Thali M, Bukovsky A, Kondo E, et al. Functional association of cyclophilin A with HIV-1 virions. *Nature.* Nov 24 1994;372(6504):363-365.
66. An P, Wang LH, Hutcheson-Dilks H, et al. Regulatory polymorphisms in the cyclophilin A gene, PPIA, accelerate progression to AIDS. *PLoS pathogens.* Jun 2007;3(6):e88.
67. Liao Z, Roos JW, Hildreth JE. Increased infectivity of HIV type 1 particles bound to cell surface and solid-phase ICAM-1 and VCAM-1 through acquired adhesion molecules LFA-1 and VLA-4. *AIDS Res Hum Retroviruses.* Mar 1 2000;16(4):355-366.
68. Hashimoto-Tane A, Yokosuka T, Ishihara C, Sakuma M, Kobayashi W, Saito T. T-cell receptor microclusters critical for T-cell activation are formed independently of lipid raft clustering. *Mol Cell Biol.* Jul 2010;30(14):3421-3429.
69. Esser MT, Graham DR, Coren LV, et al. Differential incorporation of CD45, CD80 (B7-1), CD86 (B7-2), and major histocompatibility complex class I and II molecules into human immunodeficiency virus type 1 virions and microvesicles: implications for viral pathogenesis and immune regulation. *J Virol.* Jul 2001;75(13):6173-6182.
70. Mueller DL. T cells: A proliferation of costimulatory molecules. *Curr Biol.* Mar 23 2000;10(6):R227-230.
71. Giguere JF, Diou J, Madrenas J, Tremblay MJ. Virus attachment and replication are promoted after acquisition of host CD28 and CD152 by HIV-1. *The Journal of infectious diseases.* Oct 1 2005;192(7):1265-1268.
72. Grohmann U, Orabona C, Fallarino F, et al. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat Immunol.* Nov 2002;3(11):1097-1101.
73. Boasso A, Herbeuval JP, Hardy AW, Winkler C, Shearer GM. Regulation of indoleamine 2,3-dioxygenase and tryptophanyl-tRNA-synthetase by CTLA-4-Fc in human CD4+ T cells. *Blood.* Feb 15 2005;105(4):1574-1581.
74. Chertova E, Chertov O, Coren LV, et al. Proteomic and biochemical analysis of purified human immunodeficiency virus type 1 produced from infected monocyte-derived macrophages. *J Virol.* Sep 2006;80(18):9039-9052.
75. Cantin R, Fortin JF, Tremblay M. The amount of host HLA-DR proteins acquired by HIV-1 is virus strain- and cell type-specific. *Virology.* Apr 15 1996;218(2):372-381.
76. Guo MM, Hildreth JE. HIV acquires functional adhesion receptors from host cells. *AIDS Res Hum Retroviruses.* Sep 1995;11(9):1007-1013.
77. Gomez MB, Hildreth JE. Antibody to adhesion molecule LFA-1 enhances plasma neutralization of human immunodeficiency virus type 1. *J Virol.* Aug 1995;69(8):4628-4632.
78. Brenchley JM, Ruff LE, Casazza JP, Koup RA, Price DA, Douek DC. Preferential infection shortens the life span of human immunodeficiency virus-specific CD4+ T cells in vivo. *J Virol.* Jul 2006;80(14):6801-6809.
79. Douek DC, Brenchley JM, Betts MR, et al. HIV preferentially infects HIV-specific CD4+ T cells. *Nature.* May 2 2002;417(6884):95-98.
80. Moris A, Pajot A, Blanchet F, Guivel-Benhassine F, Salcedo M, Schwartz O. Dendritic cells and HIV-specific CD4+ T cells: HIV antigen presentation, T-cell activation, and viral transfer. *Blood.* Sep 1 2006;108(5):1643-1651.

81. Giguere JF, Bounou S, Paquette JS, Madrenas J, Tremblay MJ. Insertion of host-derived costimulatory molecules CD80 (B7.1) and CD86 (B7.2) into human immunodeficiency virus type 1 affects the virus life cycle. *J Virol.* Jun 2004;78(12):6222-6232.
82. Roy J, Martin G, Giguere JF, Belanger D, Petrin M, Tremblay MJ. HIV type 1 can act as an APC upon acquisition from the host cell of peptide-loaded HLA-DR and CD86 molecules. *J Immunol.* Apr 15 2005;174(8):4779-4788.
83. Hunt PW. Role of immune activation in HIV pathogenesis. *Current HIV/AIDS reports.* Feb 2007;4(1):42-47.
84. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature.* Feb 9 2006;439(7077):682-687.
85. Day CL, Kaufmann DE, Kiepiela P, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature.* Sep 21 2006;443(7109):350-354.
86. Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *The Journal of experimental medicine.* Oct 2 2006;203(10):2223-2227.
87. Hofmeyer KA, Jeon H, Zang X. The PD-1/PD-L1 (B7-H1) pathway in chronic infection-induced cytotoxic T lymphocyte exhaustion. *Journal of biomedicine & biotechnology.* 2011;2011:451694.
88. Kaufmann DE, Walker BD. PD-1 and CTLA-4 inhibitory cosignaling pathways in HIV infection and the potential for therapeutic intervention. *J Immunol.* May 15 2009;182(10):5891-5897.
89. Porichis F, Kaufmann DE. Role of PD-1 in HIV pathogenesis and as target for therapy. *Current HIV/AIDS reports.* Mar 2012;9(1):81-90.
90. Boasso A, Vaccari M, Hryniewicz A, et al. Regulatory T-cell markers, indoleamine 2,3-dioxygenase, and virus levels in spleen and gut during progressive simian immunodeficiency virus infection. *J Virol.* Nov 2007;81(21):11593-11603.
91. Fuchs D, Forsman A, Hagberg L, et al. Immune activation and decreased tryptophan in patients with HIV-1 infection. *Journal of interferon research.* Dec 1990;10(6):599-603.
92. Kaufmann DE, Kavanagh DG, Pereyra F, et al. Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat Immunol.* Nov 2007;8(11):1246-1254.
93. Trabattoni D, Saresella M, Biasin M, et al. B7-H1 is up-regulated in HIV infection and is a novel surrogate marker of disease progression. *Blood.* Apr 1 2003;101(7):2514-2520.
94. Wang X, Zhang Z, Zhang S, et al. B7-H1 up-regulation impairs myeloid DC and correlates with disease progression in chronic HIV-1 infection. *Eur J Immunol.* Nov 2008;38(11):3226-3236.
95. Santos S, Obukhov Y, Nekhai S, Bukrinsky M, Iordanskiy S. Virus-producing cells determine the host protein profiles of HIV-1 virion cores. *Retrovirology.* 2012;9:65.
96. Roberts BD, Butera ST. Host protein incorporation is conserved among diverse HIV-1 subtypes. *AIDS.* Feb 25 1999;13(3):425-427.

97. Meyer zum Bueschenfelde CO, Unternaehrer J, Mellman I, Bottomly K. Regulated recruitment of MHC class II and costimulatory molecules to lipid rafts in dendritic cells. *J Immunol.* Nov 15 2004;173(10):6119-6124.
98. Hiltbold EM, Poloso NJ, Roche PA. MHC class II-peptide complexes and APC lipid rafts accumulate at the immunological synapse. *J Immunol.* Feb 1 2003;170(3):1329-1338.
99. von Schwedler UK, Stuchell M, Muller B, et al. The protein network of HIV budding. *Cell.* Sep 19 2003;114(6):701-713.
100. Graham DR, Mitsak MJ, Elliott ST, et al. Two-dimensional gel-based approaches for the assessment of N-Linked and O-GlcNAc glycosylation in human and simian immunodeficiency viruses. *Proteomics.* Dec 2008;8(23-24):4919-4930.
101. Hioe CE, Bastiani L, Hildreth JE, Zolla-Pazner S. Role of cellular adhesion molecules in HIV type 1 infection and their impact on virus neutralization. *AIDS Res Hum Retroviruses.* Oct 1998;14 Suppl 3:S247-254.
102. Bess JW, Jr., Gorelick RJ, Bosche WJ, Henderson LE, Arthur LO. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. *Virology.* Mar 31 1997;230(1):134-144.
103. Gluschankof P, Mondor I, Gelderblom HR, Sattentau QJ. Cell membrane vesicles are a major contaminant of gradient-enriched human immunodeficiency virus type-1 preparations. *Virology.* Mar 31 1997;230(1):125-133.
104. Ott DE. Purification of HIV-1 virions by subtilisin digestion or CD45 immunoaffinity depletion for biochemical studies. *Methods Mol Biol.* 2009;485:15-25.
105. Ott DE, Coren LV, Kane BP, et al. Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. *J Virol.* Nov 1996;70(11):7734-7743.
106. Ott DE, Coren LV, Johnson DG, et al. Actin-binding cellular proteins inside human immunodeficiency virus type 1. *Virology.* Jan 5 2000;266(1):42-51.
107. Cantin R, Diou J, Belanger D, Tremblay AM, Gilbert C. Discrimination between exosomes and HIV-1: purification of both vesicles from cell-free supernatants. *J Immunol Methods.* Sep 30 2008;338(1-2):21-30.
108. Guo Y, Singleton PA, Rowshan A, et al. Quantitative proteomics analysis of human endothelial cell membrane rafts: evidence of MARCKS and MRP regulation in the sphingosine 1-phosphate-induced barrier enhancement. *Mol Cell Proteomics.* Apr 2007;6(4):689-696.
109. Sheng S, Chen D, Van Eyk JE. Multidimensional liquid chromatography separation of intact proteins by chromatographic focusing and reversed phase of the human serum proteome: optimization and protein database. *Mol Cell Proteomics.* Jan 2006;5(1):26-34.
110. McDonald T, Sheng S, Stanley B, et al. Expanding the subproteome of the inner mitochondria using protein separation technologies: one- and two-dimensional liquid chromatography and two-dimensional gel electrophoresis. *Mol Cell Proteomics.* Dec 2006;5(12):2392-2411.
111. Edwards N, Wu X, Tseng C-W. An Unsupervised, Model-Free, Machine-Learning Combiner for Peptide Identifications from Tandem Mass Spectra. *Clinical Proteomics.* 2009;5(1):23-36.

112. Parish LA, Colquhoun DR, Ubaida Mohien C, Lyashkov AE, Graham DR, Dinglasan RR. Ookinete-interacting proteins on the microvillar surface are partitioned into detergent resistant membranes of Anopheles gambiae midguts. *J Proteome Res.* Nov 4 2011;10(11):5150-5162.
113. Hall M, Frank E, Holmes G, Pfahringer B, Reutemann P, Witten IH. The WEKA Data Mining Software: An Update. *SIGKDD Explorations Newslett.* 2009;11(1):10-18.
114. Ubaida Mohien C, Hartler J, Breitwieser F, et al. MASPECTRAS 2: An integration and analysis platform for proteomic data. *Proteomics.* Jul 2010;10(14):2719-2722.
115. Taylor CF, Paton NW, Lilley KS, et al. The minimum information about a proteomics experiment (MIAPE). *Nat Biotechnol.* Aug 2007;25(8):887-893.
116. Vizcaino JA, Cote R, Reisinger F, et al. The Proteomics Identifications database: 2010 update. *Nucleic Acids Res.* Jan 2010;38(Database issue):D736-742.
117. Montojo J, Zuberi K, Rodriguez H, et al. GeneMANIA Cytoscape plugin: fast gene function predictions on the desktop. *Bioinformatics.* Nov 15 2010;26(22):2927-2928.
118. Ptak RG, Fu W, Sanders-Beer BE, et al. Cataloguing the HIV type 1 human protein interaction network. *AIDS Res Hum Retroviruses.* Dec 2008;24(12):1497-1502.
119. Fu W, Sanders-Beer BE, Katz KS, Maglott DR, Pruitt KD, Ptak RG. Human immunodeficiency virus type 1, human protein interaction database at NCBI. *Nucleic Acids Res.* Jan 2009;37(Database issue):D417-422.
120. Pinney JW, Dickerson JE, Fu W, Sanders-Beer BE, Ptak RG, Robertson DL. HIV-host interactions: a map of viral perturbation of the host system. *AIDS.* Mar 13 2009;23(5):549-554.
121. Breitwieser FP, Muller A, Dayon L, et al. General statistical modeling of data from protein relative expression isobaric tags. *J Proteome Res.* Jun 3 2011;10(6):2758-2766.
122. Yu X, Harris SL, Levine AJ. The regulation of exosome secretion: a novel function of the p53 protein. *Cancer Res.* May 1 2006;66(9):4795-4801.
123. Welton JL, Khanna S, Giles PJ, et al. Proteomics analysis of bladder cancer exosomes. *Mol Cell Proteomics.* Jun 2010;9(6):1324-1338.
124. Buschow SI, van Balkom BW, Aalberts M, Heck AJ, Wauben M, Stoorvogel W. MHC class II-associated proteins in B-cell exosomes and potential functional implications for exosome biogenesis. *Immunol Cell Biol.* Nov-Dec 2010;88(8):851-856.
125. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem.* Aug 7 1998;273(32):20121-20127.
126. Staubach S, Razawi H, Hanisch FG. Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7. *Proteomics.* May 2009;9(10):2820-2835.
127. Salter RD, Howell DN, Cresswell P. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics.* 1985;21(3):235-246.

128. Klyushnenkova EN, Li L, Armitage RJ, Choi YS. CD48 delivers an accessory signal for CD40-mediated activation of human B cells. *Cell Immunol.* Nov 25 1996;174(1):90-98.
129. Ubaida Mohien C, Colquhoun DR, Mathias DK, et al. A Bioinformatics Approach for Integrated Transcriptomic and Proteomic Comparative Analyses of Model and Non-sequenced Anopheline Vectors of Human Malaria Parasites. *Mol Cell Proteomics.* Jan 2013;12(1):120-131.
130. Charrin S, Manie S, Thiele C, et al. A physical and functional link between cholesterol and tetraspanins. *Eur J Immunol.* Sep 2003;33(9):2479-2489.
131. Krementsov DN, Weng J, Lambele M, Roy NH, Thali M. Tetraspanins regulate cell-to-cell transmission of HIV-1. *Retrovirology.* 2009;6:64.
132. Weng J, Krementsov DN, Khurana S, Roy NH, Thali M. Formation of syncytia is repressed by tetraspanins in human immunodeficiency virus type 1-producing cells. *J Virol.* Aug 2009;83(15):7467-7474.
133. Grigorov B, Attuil-Audenis V, Perugi F, et al. A role for CD81 on the late steps of HIV-1 replication in a chronically infected T cell line. *Retrovirology.* 2009;6:28.
134. Yanez-Mo M, Barreiro O, Gordon-Alonso M, Sala-Valdes M, Sanchez-Madrid F. Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. *Trends Cell Biol.* Sep 2009;19(9):434-446.
135. Beekman JM, Coffer PJ. The ins and outs of syntenin, a multifunctional intracellular adaptor protein. *J Cell Sci.* May 1 2008;121(Pt 9):1349-1355.
136. Johnson P, Ruffell B. CD44 and its role in inflammation and inflammatory diseases. *Inflamm Allergy Drug Targets.* Jul 2009;8(3):208-220.
137. Guo MM, Hildreth JE. HIV-induced loss of CD44 expression in monocytic cell lines. *J Immunol.* Aug 15 1993;151(4):2225-2236.
138. Luban J. Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. *J Virol.* Feb 2007;81(3):1054-1061.
139. Strebel K, Luban J, Jeang KT. Human cellular restriction factors that target HIV-1 replication. *BMC Med.* 2009;7:48.
140. Cordelier P, Strayer DS. Mechanisms of alpha1-antitrypsin inhibition of cellular serine proteases and HIV-1 protease that are essential for HIV-1 morphogenesis. *Biochim Biophys Acta.* Jul 30 2003;1638(3):197-207.
141. Shapiro L, Pott GB, Ralston AH. Alpha-1-antitrypsin inhibits human immunodeficiency virus type 1. *FASEB J.* Jan 2001;15(1):115-122.
142. Anderson ED, Thomas L, Hayflick JS, Thomas G. Inhibition of HIV-1 gp160-dependent membrane fusion by a furin-directed alpha 1-antitrypsin variant. *J Biol Chem.* Nov 25 1993;268(33):24887-24891.
143. George M, Ying G, Rainey MA, et al. Shared as well as distinct roles of EHD proteins revealed by biochemical and functional comparisons in mammalian cells and C. elegans. *BMC Cell Biol.* 2007;8:3.
144. Sharma M, Naslavsky N, Caplan S. A role for EHD4 in the regulation of early endosomal transport. *Traffic.* Jun 2008;9(6):995-1018.
145. Salcini AE, Confalonieri S, Doria M, et al. Binding specificity and in vivo targets of the EH domain, a novel protein-protein interaction module. *Genes Dev.* Sep 1 1997;11(17):2239-2249.

146. Joshi P, Stoddart CA. Impaired infectivity of ritonavir-resistant HIV is rescued by heat shock protein 90AB1. *J Biol Chem.* Jul 15 2011;286(28):24581-24592.
147. Vozzolo L, Loh B, Gane PJ, et al. Gyrase B inhibitor impairs HIV-1 replication by targeting Hsp90 and the capsid protein. *J Biol Chem.* Dec 10 2010;285(50):39314-39328.
148. Ryzhova EV, Vos RM, Albright AV, Harrist AV, Harvey T, Gonzalez-Scarano F. Annexin 2: a novel human immunodeficiency virus type 1 Gag binding protein involved in replication in monocyte-derived macrophages. *J Virol.* Mar 2006;80(6):2694-2704.
149. Harrist AV, Ryzhova EV, Harvey T, Gonzalez-Scarano F. Anx2 interacts with HIV-1 Gag at phosphatidylinositol (4,5) bisphosphate-containing lipid rafts and increases viral production in 293T cells. *PLoS One.* 2009;4(3):e5020.
150. Rai T, Mosoian A, Resh MD. Annexin 2 is not required for human immunodeficiency virus type 1 particle production but plays a cell type-dependent role in regulating infectivity. *J Virol.* Oct 2010;84(19):9783-9792.
151. Trubey CM, Chertova E, Coren LV, et al. Quantitation of HLA class II protein incorporated into human immunodeficiency type 1 virions purified by anti-CD45 immunoaffinity depletion of microvesicles. *J Virol.* Dec 2003;77(23):12699-12709.
152. Denard J, Rundwasser S, Laroudie N, et al. Quantitative proteomic analysis of lentiviral vectors using 2-DE. *Proteomics.* Jul 2009;9(14):3666-3676.
153. Falick AM, Lane WS, Lilley KS, et al. ABRF-PRG07: advanced quantitative proteomics study. *J Biomol Tech.* Apr 2011;22(1):21-26.
154. Bell AW, Deutsch EW, Au CE, et al. A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. *Nat Methods.* Jun 2009;6(6):423-430.
155. Stipp CS, Kolesnikova TV, Hemler ME. Functional domains in tetraspanin proteins. *Trends in biochemical sciences.* Feb 2003;28(2):106-112.
156. Maecker HT, Todd SC, Levy S. The tetraspanin superfamily: molecular facilitators. *FASEB J.* May 1997;11(6):428-442.
157. Fanaei M, Monk PN, Partridge LJ. The role of tetraspanins in fusion. *Biochemical Society transactions.* Apr 2011;39(2):524-528.
158. Takeda Y, Tachibana I, Miyado K, et al. Tetraspanins CD9 and CD81 function to prevent the fusion of mononuclear phagocytes. *J Cell Biol.* Jun 9 2003;161(5):945-956.
159. Bailey RL, Herbert JM, Khan K, Heath VL, Bicknell R, Tomlinson MG. The emerging role of tetraspanin microdomains on endothelial cells. *Biochemical Society transactions.* Dec 2011;39(6):1667-1673.
160. Barreiro O, Yanez-Mo M, Sala-Valdes M, et al. Endothelial tetraspanin microdomains regulate leukocyte firm adhesion during extravasation. *Blood.* Apr 1 2005;105(7):2852-2861.
161. Berditchevski F. Complexes of tetraspanins with integrins: more than meets the eye. *J Cell Sci.* Dec 2001;114(Pt 23):4143-4151.
162. Feigelson SW, Grabovsky V, Shamri R, Levy S, Alon R. The CD81 tetraspanin facilitates instantaneous leukocyte VLA-4 adhesion strengthening to vascular cell

adhesion molecule 1 (VCAM-1) under shear flow. *J Biol Chem.* Dec 19 2003;278(51):51203-51212.

163. Gilsanz A, Sanchez-Martin L, Gutierrez-Lopez MD, et al. ALCAM/CD166 adhesive function is regulated by the tetraspanin CD9. *Cellular and molecular life sciences : CMLS.* Feb 2013;70(3):475-493.

164. Green LR, Monk PN, Partridge LJ, Morris P, Gorringe AR, Read RC. Cooperative role for tetraspanins in adhesin-mediated attachment of bacterial species to human epithelial cells. *Infection and immunity.* Jun 2011;79(6):2241-2249.

165. He B, Liu L, Cook GA, Grgurevich S, Jennings LK, Zhang XA. Tetraspanin CD82 attenuates cellular morphogenesis through down-regulating integrin alpha6-mediated cell adhesion. *J Biol Chem.* Feb 4 2005;280(5):3346-3354.

166. VanCompernolle SE, Levy S, Todd SC. Anti-CD81 activates LFA-1 on T cells and promotes T cell-B cell collaboration. *Eur J Immunol.* Mar 2001;31(3):823-831.

167. Bari R, Guo Q, Xia B, et al. Tetraspanins regulate the protrusive activities of cell membrane. *Biochem Biophys Res Commun.* Dec 2 2011;415(4):619-626.

168. Lee SA, Park KH, Lee JW. Modulation of signaling between TM4SF5 and integrins in tumor microenvironment. *Frontiers in bioscience : a journal and virtual library.* 2011;16:1752-1758.

169. Levy S, Shoham T. The tetraspanin web modulates immune-signalling complexes. *Nature reviews. Immunology.* Feb 2005;5(2):136-148.

170. Little KD, Hemler ME, Stipp CS. Dynamic regulation of a GPCR-tetraspanin-G protein complex on intact cells: central role of CD81 in facilitating GPR56-Galpha q/11 association. *Mol Biol Cell.* May 2004;15(5):2375-2387.

171. Petersen SH, Odintsova E, Haigh TA, Rickinson AB, Taylor GS, Berditchevski F. The role of tetraspanin CD63 in antigen presentation via MHC class II. *Eur J Immunol.* Sep 2011;41(9):2556-2561.

172. Rana S, Zoller M. Exosome target cell selection and the importance of exosomal tetraspanins: a hypothesis. *Biochemical Society transactions.* Apr 2011;39(2):559-562.

173. Verweij FJ, van Eijndhoven MA, Hopmans ES, et al. LMP1 association with CD63 in endosomes and secretion via exosomes limits constitutive NF-kappaB activation. *The EMBO journal.* Jun 1 2011;30(11):2115-2129.

174. Bertaux C, Dragic T. Different domains of CD81 mediate distinct stages of hepatitis C virus pseudoparticle entry. *J Virol.* May 2006;80(10):4940-4948.

175. Farquhar MJ, Hu K, Harris HJ, et al. Hepatitis C virus induces CD81 and claudin-1 endocytosis. *J Virol.* Apr 2012;86(8):4305-4316.

176. Diao J, Pantua H, Ngu H, et al. Hepatitis C virus induces epidermal growth factor receptor activation via CD81 binding for viral internalization and entry. *J Virol.* Oct 2012;86(20):10935-10949.

177. Martin F, Roth DM, Jans DA, et al. Tetraspanins in viral infections: a fundamental role in viral biology? *J Virol.* Sep 2005;79(17):10839-10851.

178. Mazurov D, Heidecker G, Derse D. The inner loop of tetraspanins CD82 and CD81 mediates interactions with human T cell lymphotrophic virus type 1 Gag protein. *J Biol Chem.* Feb 9 2007;282(6):3896-3903.

179. Mazurov D, Heidecker G, Derse D. HTLV-1 Gag protein associates with CD82 tetraspanin microdomains at the plasma membrane. *Virology.* Mar 1 2006;346(1):194-204.
180. Petrovic D, Stamataki Z, Dempsey E, et al. Hepatitis C virus targets the T cell secretory machinery as a mechanism of immune evasion. *Hepatology.* Jun 2011;53(6):1846-1853.
181. Pique C, Lagaudriere-Gesbert C, Delamarre L, Rosenberg AR, Conjeaud H, Dokhelar MC. Interaction of CD82 tetraspanin proteins with HTLV-1 envelope glycoproteins inhibits cell-to-cell fusion and virus transmission. *Virology.* Oct 25 2000;276(2):455-465.
182. Rajesh S, Sridhar P, Tews BA, et al. Structural basis of ligand interactions of the large extracellular domain of tetraspanin CD81. *J Virol.* Sep 2012;86(18):9606-9616.
183. Silvie O, Charrin S, Billard M, et al. Cholesterol contributes to the organization of tetraspanin-enriched microdomains and to CD81-dependent infection by malaria sporozoites. *J Cell Sci.* May 15 2006;119(Pt 10):1992-2002.
184. Zhu YZ, Luo Y, Cao MM, et al. Significance of palmitoylation of CD81 on its association with tetraspanin-enriched microdomains and mediating hepatitis C virus cell entry. *Virology.* Aug 1 2012;429(2):112-123.
185. Akuthota P, Melo RC, Spencer LA, Weller PF. MHC Class II and CD9 in human eosinophils localize to detergent-resistant membrane microdomains. *American journal of respiratory cell and molecular biology.* Feb 2012;46(2):188-195.
186. Jones EL, Demaria MC, Wright MD. Tetraspanins in cellular immunity. *Biochemical Society transactions.* Apr 2011;39(2):506-511.
187. Kobayashi H, Hosono O, Iwata S, et al. The tetraspanin CD9 is preferentially expressed on the human CD4(+)CD45RA+ naive T cell population and is involved in T cell activation. *Clinical and experimental immunology.* Jul 2004;137(1):101-108.
188. Peng WM, Yu CF, Kolanus W, et al. Tetraspanins CD9 and CD81 are molecular partners of trimeric FcvarepsilonRI on human antigen-presenting cells. *Allergy.* May 2011;66(5):605-611.
189. Quast T, Eppler F, Semmling V, et al. CD81 is essential for the formation of membrane protrusions and regulates Rac1-activation in adhesion-dependent immune cell migration. *Blood.* Aug 18 2011;118(7):1818-1827.
190. van Spriel AB. Tetraspanins in the humoral immune response. *Biochemical Society transactions.* Apr 2011;39(2):512-517.
191. Powner D, Kopp PM, Monkley SJ, Critchley DR, Berditchevski F. Tetraspanin CD9 in cell migration. *Biochemical Society transactions.* Apr 2011;39(2):563-567.
192. Richardson MM, Jennings LK, Zhang XA. Tetraspanins and tumor progression. *Clinical & experimental metastasis.* Mar 2011;28(3):261-270.
193. Scales TM, Jayo A, Obara B, et al. alpha3beta1 integrins regulate CD151 complex assembly and membrane dynamics in carcinoma cells within 3D environments. *Oncogene.* Sep 17 2012.

194. Lapalombella R, Yeh YY, Wang L, et al. Tetraspanin CD37 directly mediates transduction of survival and apoptotic signals. *Cancer cell.* May 15 2012;21(5):694-708.
195. Hemler ME. Specific tetraspanin functions. *J Cell Biol.* Dec 24 2001;155(7):1103-1107.
196. Hemler ME. Tetraspanin functions and associated microdomains. *Nature reviews. Molecular cell biology.* Oct 2005;6(10):801-811.
197. Delaguillaumie A, Harriague J, Kohanna S, et al. Tetraspanin CD82 controls the association of cholesterol-dependent microdomains with the actin cytoskeleton in T lymphocytes: relevance to co-stimulation. *J Cell Sci.* Oct 15 2004;117(Pt 22):5269-5282.
198. Le Naour F, Andre M, Boucheix C, Rubinstein E. Membrane microdomains and proteomics: lessons from tetraspanin microdomains and comparison with lipid rafts. *Proteomics.* Dec 2006;6(24):6447-6454.
199. Charrin S, le Naour F, Silvie O, Milhiet PE, Boucheix C, Rubinstein E. Lateral organization of membrane proteins: tetraspanins spin their web. *Biochem J.* Jun 1 2009;420(2):133-154.
200. Douglass AD, Vale RD. Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. *Cell.* Jun 17 2005;121(6):937-950.
201. Waheed AA, Freed EO. Lipids and membrane microdomains in HIV-1 replication. *Virus research.* Aug 2009;143(2):162-176.
202. Cramer EM, Berger G, Berndt MC. Platelet alpha-granule and plasma membrane share two new components: CD9 and PECAM-1. *Blood.* Sep 15 1994;84(6):1722-1730.
203. Brisson C, Azorsa DO, Jennings LK, Moog S, Cazenave JP, Lanza F. Co-localization of CD9 and GPIIb-IIIa (alpha IIb beta 3 integrin) on activated platelet pseudopods and alpha-granule membranes. *The Histochemical journal.* Feb 1997;29(2):153-165.
204. Heijnen HF, Debili N, Vainchencker W, Breton-Gorius J, Geuze HJ, Sixma JJ. Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. *Blood.* Apr 1 1998;91(7):2313-2325.
205. Nishibori M, Cham B, McNicol A, Shalev A, Jain N, Gerrard JM. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky-Pudlak syndrome, and appears identical to granulophysin. *The Journal of clinical investigation.* Apr 1993;91(4):1775-1782.
206. Nieuwenhuis HK, van Oosterhout JJ, Rozemuller E, van Iwaarden F, Sixma JJ. Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood.* Sep 1987;70(3):838-845.
207. Peters PJ, Neefjes JJ, Oorschot V, Ploegh HL, Geuze HJ. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature.* Feb 21 1991;349(6311):669-676.
208. Peters PJ, Borst J, Oorschot V, et al. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *The Journal of experimental medicine.* May 1 1991;173(5):1099-1109.

209. Kobayashi T, Vischer UM, Rosnoblet C, et al. The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. *Mol Biol Cell.* May 2000;11(5):1829-1843.
210. van der Wel NN, Sugita M, Fluitsma DM, et al. CD1 and major histocompatibility complex II molecules follow a different course during dendritic cell maturation. *Mol Biol Cell.* Aug 2003;14(8):3378-3388.
211. Kobayashi T, Beuchat MH, Chevallier J, et al. Separation and characterization of late endosomal membrane domains. *J Biol Chem.* Aug 30 2002;277(35):32157-32164.
212. Garcia E, Pion M, Pelchen-Matthews A, et al. HIV-1 trafficking to the dendritic cell-T-cell infectious synapse uses a pathway of tetraspanin sorting to the immunological synapse. *Traffic.* Jun 2005;6(6):488-501.
213. Pelchen-Matthews A, Kramer B, Marsh M. Infectious HIV-1 assembles in late endosomes in primary macrophages. *J Cell Biol.* Aug 4 2003;162(3):443-455.
214. Hammond C, Denzin LK, Pan M, Griffith JM, Geuze HJ, Cresswell P. The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. *J Immunol.* Oct 1 1998;161(7):3282-3291.
215. Deneka M, Pelchen-Matthews A, Byland R, Ruiz-Mateos E, Marsh M. In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. *J Cell Biol.* Apr 23 2007;177(2):329-341.
216. Jolly C, Sattentau QJ. Human immunodeficiency virus type 1 assembly, budding, and cell-cell spread in T cells take place in tetraspanin-enriched plasma membrane domains. *J Virol.* Aug 2007;81(15):7873-7884.
217. Nydegger S, Khurana S, Krementsov DN, Foti M, Thali M. Mapping of tetraspanin-enriched microdomains that can function as gateways for HIV-1. *J Cell Biol.* Jun 5 2006;173(5):795-807.
218. Gordon-Alonso M, Sala-Valdes M, Rocha-Perugini V, et al. EWI-2 association with alpha-actinin regulates T cell immune synapses and HIV viral infection. *J Immunol.* Jul 15 2012;189(2):689-700.
219. Gordon-Alonso M, Yanez-Mo M, Barreiro O, et al. Tetraspanins CD9 and CD81 modulate HIV-1-induced membrane fusion. *J Immunol.* Oct 15 2006;177(8):5129-5137.
220. Li G, Dziuba N, Friedrich B, Murray JL, Ferguson MR. A post-entry role for CD63 in early HIV-1 replication. *Virology.* Apr 10 2011;412(2):315-324.
221. Ho SH, Martin F, Higginbottom A, et al. Recombinant extracellular domains of tetraspanin proteins are potent inhibitors of the infection of macrophages by human immunodeficiency virus type 1. *J Virol.* Jul 2006;80(13):6487-6496.
222. Meroni L, Milazzo L, Menzaghi B, et al. Altered expression of the tetraspanin CD81 on B and T lymphocytes during HIV-1 infection. *Clinical and experimental immunology.* Jan 2007;147(1):53-59.
223. Meerschaert K, Bruyneel E, De Wever O, et al. The tandem PDZ domains of syntenin promote cell invasion. *Experimental cell research.* May 15 2007;313(9):1790-1804.

224. Batonick M, Favre M, Boge M, Spearman P, Honing S, Thali M. Interaction of HIV-1 Gag with the clathrin-associated adaptor AP-2. *Virology.* Nov 25 2005;342(2):190-200.
225. Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annual review of biochemistry.* 2003;72:395-447.
226. Rous BA, Reaves BJ, Ihrke G, et al. Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. *Mol Biol Cell.* Mar 2002;13(3):1071-1082.
227. Dong X, Li H, Derdowski A, et al. AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly. *Cell.* Mar 11 2005;120(5):663-674.
228. Kyere SK, Mercredi PY, Dong X, Spearman P, Summers MF. The HIV-1 matrix protein does not interact directly with the protein interactive domain of AP-3delta. *Virus research.* Nov 2012;169(2):411-414.
229. Andre M, Le Caer JP, Greco C, et al. Proteomic analysis of the tetraspanin web using LC-ESI-MS/MS and MALDI-FTICR-MS. *Proteomics.* Mar 2006;6(5):1437-1449.
230. Sala-Valdes M, Ursa A, Charrin S, et al. EWI-2 and EWI-F link the tetraspanin web to the actin cytoskeleton through their direct association with ezrin-radixin-moesin proteins. *J Biol Chem.* Jul 14 2006;281(28):19665-19675.
231. Zhang XA, Bontrager AL, Hemler ME. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. *J Biol Chem.* Jul 6 2001;276(27):25005-25013.
232. Latysheva N, Muratov G, Rajesh S, et al. Syntenin-1 is a new component of tetraspanin-enriched microdomains: mechanisms and consequences of the interaction of syntenin-1 with CD63. *Mol Cell Biol.* Oct 2006;26(20):7707-7718.
233. Baietti MF, Zhang Z, Mortier E, et al. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nature cell biology.* Jul 2012;14(7):677-685.
234. Rodrigues AF, Guerreiro MR, Santiago VM, et al. Down-regulation of CD81 tetraspanin in human cells producing retroviral-based particles: tailoring vector composition. *Biotechnology and bioengineering.* Nov 2011;108(11):2623-2633.
235. Stamer WD, Hoffman EA, Luther JM, Hachey DL, Schey KL. Protein profile of exosomes from trabecular meshwork cells. *Journal of proteomics.* May 16 2011;74(6):796-804.
236. Segura MM, Garnier A, Di Falco MR, et al. Identification of host proteins associated with retroviral vector particles by proteomic analysis of highly purified vector preparations. *J Virol.* Feb 2008;82(3):1107-1117.
237. Fujii K, Hurley JH, Freed EO. Beyond Tsg101: the role of Alix in 'ESCRTing' HIV-1. *Nature reviews. Microbiology.* Dec 2007;5(12):912-916.
238. Chou T, Kim KS, Oster G. Statistical thermodynamics of membrane bending-mediated protein-protein attractions. *Biophysical journal.* Mar 2001;80(3):1075-1087.
239. Martin-Serrano J, Neil SJ. Host factors involved in retroviral budding and release. *Nature reviews. Microbiology.* Jul 2011;9(7):519-531.
240. Katsov K, Muller M, Schick M. Field theoretic study of bilayer membrane fusion. I. Hemifusion mechanism. *Biophysical journal.* Nov 2004;87(5):3277-3290.

241. Wieslander A, Christiansson A, Rilfors L, Lindblom G. Lipid bilayer stability in membranes. Regulation of lipid composition in Acholeplasma laidlawii as governed by molecular shape. *Biochemistry.* Aug 5 1980;19(16):3650-3655.
242. Morein S, Andersson A, Rilfors L, Lindblom G. Wild-type Escherichia coli cells regulate the membrane lipid composition in a "window" between gel and non-lamellar structures. *J Biol Chem.* Mar 22 1996;271(12):6801-6809.
243. Schroeder C. Cholesterol-binding viral proteins in virus entry and morphogenesis. *Sub-cellular biochemistry.* 2010;51:77-108.
244. Kamiyama H, Yoshii H, Tanaka Y, Sato H, Yamamoto N, Kubo Y. Raft localization of CXCR4 is primarily required for X4-tropic human immunodeficiency virus type 1 infection. *Virology.* Mar 30 2009;386(1):23-31.
245. Cardaba CM, Kerr JS, Mueller A. CCR5 internalisation and signalling have different dependence on membrane lipid raft integrity. *Cellular signalling.* Sep 2008;20(9):1687-1694.
246. Carter GC, Bernstone L, Sangani D, Bee JW, Harder T, James W. HIV entry in macrophages is dependent on intact lipid rafts. *Virology.* Mar 30 2009;386(1):192-202.
247. Abbas W, Herbein G. Plasma membrane signaling in HIV-1 infection. *Biochim Biophys Acta.* Jun 24 2013.
248. Feire AL, Koss H, Compton T. Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. *Proc Natl Acad Sci U S A.* Oct 26 2004;101(43):15470-15475.
249. Haspot F, Lavault A, Sinzger C, et al. Human cytomegalovirus entry into dendritic cells occurs via a macropinocytosis-like pathway in a pH-independent and cholesterol-dependent manner. *PLoS One.* 2012;7(4):e34795.
250. Ogembo JG, Kannan L, Ghiran I, et al. Human complement receptor type 1/CD35 is an Epstein-Barr Virus receptor. *Cell reports.* Feb 21 2013;3(2):371-385.
251. Katzman RB, Longnecker R. Cholesterol-dependent infection of Burkitt's lymphoma cell lines by Epstein-Barr virus. *The Journal of general virology.* Nov 2003;84(Pt 11):2987-2992.
252. Glebe D, Urban S. Viral and cellular determinants involved in hepadnaviral entry. *World journal of gastroenterology : WJG.* Jan 7 2007;13(1):22-38.
253. Fuki IV, Meyer ME, Williams KJ. Transmembrane and cytoplasmic domains of syndecan mediate a multi-step endocytic pathway involving detergent-insoluble membrane rafts. *Biochem J.* Nov 1 2000;351 Pt 3:607-612.
254. Gianni T, Campadelli-Fiume G. alphaVbeta3-integrin relocalizes nectin1 and routes herpes simplex virus to lipid rafts. *J Virol.* Mar 2012;86(5):2850-2855.
255. Gianni T, Gatta V, Campadelli-Fiume G. {alpha}V{beta}3-integrin routes herpes simplex virus to an entry pathway dependent on cholesterol-rich lipid rafts and dynamin2. *Proc Natl Acad Sci U S A.* Dec 21 2010;107(51):22260-22265.
256. Rahn E, Petermann P, Hsu MJ, Rixon FJ, Knebel-Morsdorf D. Entry pathways of herpes simplex virus type 1 into human keratinocytes are dynamin- and cholesterol-dependent. *PLoS One.* 2011;6(10):e25464.
257. Hambleton S, Steinberg SP, Gershon MD, Gershon AA. Cholesterol dependence of varicella-zoster virion entry into target cells. *J Virol.* Jul 2007;81(14):7548-7558.

258. Chen JJ, Zhu Z, Gershon AA, Gershon MD. Mannose 6-phosphate receptor dependence of varicella zoster virus infection in vitro and in the epidermis during varicella and zoster. *Cell.* Dec 29 2004;119(7):915-926.
259. Ren X, Xue C, Kong Q, Zhang C, Bi Y, Cao Y. Proteomic analysis of purified Newcastle disease virus particles. *Proteome science.* 2012;10(1):32.
260. Shaw ML, Stone KL, Colangelo CM, Gulcicek EE, Palese P. Cellular proteins in influenza virus particles. *PLoS pathogens.* Jun 2008;4(6):e1000085.
261. Zhang C, Xue C, Li Y, et al. Profiling of cellular proteins in porcine reproductive and respiratory syndrome virus virions by proteomics analysis. *Virology journal.* 2010;7:242.

Curriculum Vitae

The Johns Hopkins University School of Medicine

Michael Linde, MS March 2014

Educational History

Ph.D., expected	2014	Program in Immunology Mentor: James E. K. Hildreth, MD, PhD	Johns Hopkins School of Medicine
M.S.	2003	Biochem. & Molec. Biology	University of Southern California
B.A.	1996	Biology/English	Emory University

Other Professional Experience

Medical Director	January 2014-Present	ClinicalMind; Jersey City, NJ
Medical Writer	October 2002-Dec. 2013	Linde Medical Writing
Medical Writer	May 1999-July 2001	Medisolutions; New York, NY
Medical Abstractor	January 1998-April 1999	Information Inc.; Bethesda, MD
Intern	August 1994-December 1994	Cable News Network; Atlanta, GA
Research Assistant	February 1994-August 1994	Emory University; Atlanta, GA

Publications, Peer Reviewed

Linde ME, Colquhoun DR, Mohien CU, Kole T, Aquino V, Cotter R, Edwards N, Hildreth JE, Graham DR (2013) The conserved set of host proteins incorporated into HIV-1 virions suggests a common egress pathway in multiple cell types. J Proteome Res 12:2045-54.

Weed M, Adams RJ, Hienz RD, Meulendyke KA, **Linde ME**, Clements JE, Mankowski JL, Zink MC (2011) SIV/Macaque Model of HIV Infection in Cocaine Users: Minimal Effects of Cocaine on Behavior, Virus Replication, and CNS Inflammation. J Neuroimmune Pharmacol 7:401-11.

Taylor HE, **Linde ME**, Khatua AK, Popik W, Hildreth JE (2011) Sterol regulatory element-binding protein 2 couples HIV-1 transcription to cholesterol homeostasis and T cell activation. J Virol 85:7699-709.

Law MJ, **Linde ME**, Chambers EJ, Oubridge C, Katsamba PS, Haworth IS, Laird-Offringa IA (2006) The Role of Positively Charged Amino Acids and Electrostatic Interactions in the Complex of U1A Protein and U1 Hairpin II RNA. Nucleic Acids Res 34(1):275-285.

Publications, Other

Linde M (2002) Should you enroll in a clinical trial? Positive Living 11(5):37-42.

Linde M and Pieribone D (2002) Reseñas de la 9na Conferencia de Retrovirus e Infecciones Oportunistas. Impacto! 1(5):22.

Linde M (2002) ‘Fine tuning’ HIV therapy: Report on the 9th Conference on Retroviruses and Opportunistic Infections. Positive Living 11(3):8-9,16,44.